EVALUATION OF ENZYMATIC ACTIVITIES IN RUMEN FLUID AND RESPECTIVE *IN VITRO* DIGESTIBILITIES

by

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DECLARATION

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NOTE

The language and style used in this thesis are in accordance with the requirements of the *Journal of Dairy Science*. This thesis represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

NOTA

Die taal en skryfstyl wat gebruik is in hierdie tesis is volgens die vereistes van die *Tydskrif vir Suiwelkunde*. Hierdie tesis verteenwoordig ‘n samevatting van manuskripte, waar elke hoofstuk as ‘n enkele entiteit bestaan, en gevolglik is herhaling van inligting tussen hoofstukke onvermydelik.

ABSTRACT

Rumen enzymatic activity studies and *in vitro* digestibility studies are potentially correlated and affected by similar factors. However, performing enzymatic activity studies parallel to *in vitro* digestibility studies is hindered by the lack of a standard method of analysis and by the lack of knowledge surrounding the variables that could potentially influence the enzymatic activity. It is known that the composition of the ruminal microbial population is responsive to changes in various natural and feed related factors, but the dynamic between the microbial population and enzymatic activity within the rumen, and factors affecting enzymatic activity are not well defined. The objective of the first experiment was to determine the effect of blending and sonication on enzymatic (amylolytic, lichenase, endoglucanase, xylanase, protease) activity and the effect of blending on *in vitro* digestibility (starch, neutral detergent fibre, nitrogen) when compared to simple filtration (control). Both blending and sonication significantly and consistently increased amylase, lichenase, endoglucanase and xylanase activity with no significant difference between the two enzyme extraction methods. For protease activity, only sonication was able to significantly increase the activity compared to the control. For the *in vitro* analysis, blending was unsuccessful in liberating amylolytic microorganism associated with the particulate fraction and might have negatively affected the microbial population responsible for neutral detergent fibre (NDF) and protein digestion. The objective of the second experiment was to determine if diurnal patterns in starch, NDF and protein digestibility and amylolytic, fibrolytic and proteolytic activity, respectively, exist. Two of the cows were restricted from feed for 8-hours overnight, and the other two continued to receive their feed *ad libitum*, to isolate but also quantify the effects of a possible different feeding behaviour at night. Rumen fluid was sampled before the morning feeding, and subsequent collections were every 4-hours for a 24-hour period. When fitted to a cosine function, all the parameters tested followed a daily pattern that was sensitive to the availability of feed overnight, although the parameters responded differently to the feeding treatment. The highest activities for amylase, lichenase, endoglucanase and

xylanase were observed at the time points subsequent to milking and feed delivery, suggesting that the cows adapted their feeding behaviour to the time of feed delivery and milking. Protease activity was unaffected by either feeding treatment or possible feeding behaviour, although when fitted to a cosine function it did display a daily pattern that was sensitive to the availability of feed overnight. The patterns displayed by *in vitro* digestibility of starch, NDF and nitrogen, across the various fluid collection time points, were much more variable than expected and could be attributed to the fact that a higher number of variables affect the final results. The acrophases observed in the enzymatic analysis did not correspond to those observed in the *in vitro* analysis. Therefore, different interpretations should be given to enzymatic activities and *in vitro* digestibility values and the time of rumen fluid collection relative to feeding time should always be included when rumen fluid is utilised for research or commercial purposes.

OPSOMMING

Rumen ensiemaktiwiteite en *in vitro* verteringstudies is potensiëel gekorreleer en beïnvloed deur soortgelyke faktore, alhoewel die uitvoering van ensiem aktiwiteitstudies in parallel met *in vitro* verteringstudies belemmer word deur die gebrek aan ‘n standaard ontledingsmetode en die nodige kennis aangaande die veranderlikes wat potensiëel die ensiemaktiwiteite kan beïnvloed. Dit is bekend dat die samestelling van die rumen mikrobepopulasie reageer op verskeie natuurlike en voerverwante faktore, maar die dinamika tussen die rumen mikrobepopulasie, ensiemaktiwiteite, en die faktore wat ensiemaktiwiteite beïnvloed word nie goed verstaan nie. Die doel van die eerste eksperiment was om die effek van vermenging en sonikasie op ensiem- (amilase, ligenase, endoglukanase, xilanase, protease) aktiwiteit en die invloed van vermenging op *in vitro* verteerbaarheid (stysel, neutraal-onoplosbare vesel, stikstof) te bepaal wanneer dit vergelyk word met eenvoudige filtrasie (kontrole). Beide metodes het amilase-, ligenase-, endoglukanase- en xilanase-aktiwiteit betekenisvol en deurlopend verhoog, met geen betekenisvolle verskil tussen die twee ensiemontrekkingsmetodes nie. Slegs sonikasie was in staat om protease-aktiwiteit betekenisvol te verhoog in vergelyking met die kontrole. Vir die *in vitro* analise was vermenging nie in staat om amilolitiese mikroörganismes wat met die partikelfraksie geassosieer is te skei nie en het moontlik die mikrobepopulasie wat verantwoordelik is vir neutraal-onoplosbare vesel (NDF)- en proteïenvertering negatief beïnvloed. Die doel van die tweede eksperiment was om vas te stel of dag-nagritme patrone onderskeidelik in stysel-, NDF- en proteïenverteerbaarheid en amilolitiese-, fibrolitiese- en proteolitiese-aktiwiteit bestaan. Twee koeie is vir 8-uur oornag gevas en die ander twee is *ad libitum* gevoer. Die doel was om die invloed van moontlike verskille in voedingsgedrag in die nag te ondersoek en indien dit bestaan, te kwantifiseer. Rumenvloeistof is versamel voor die oggend voerperiode en die daaropvolgende versamelings is elke 4-uur vir ‘n 24-uur periode herhaal. Toe die data met ‘n kosinusfunksie gepas is het al die parameters wat getoets is’n dag-nagritme patroon gevolg wat beïnvloed is deur die beskikbaarheid van voer snags. Die hoogste amilase-, ligenase-, endoglukanase-

en xilanase-aktiwiteit is waargeneem by tydpunte wat gevolg het na melking en voerlewering, wat waarskynlik beteken dat die koeie hulle voedingsgedrag aangepas het tot die tyd van melking en voerlewering. Protease-aktiwiteit is nie beïnvloed deur voer-behandeling of deur voedingsgedrag nie, alhoewel dit ‘n dag-nagritme patroon gevolg het wat beïnvloed is deur die beskikbaarheid van voer snags soos aangedui deur die kosinusfunksie. Die patrone wat waargeneem is by die *in vitro* verteerbaarheid van stysel, NDF en stikstof oor die verskeie tydpunte was meer variërend as wat verwag is en dit kan toegeskryf word aan die groter hoeveelheid veranderlikes wat die finale resultate beïnvloed het. Die akrofases wat waargeneem is in die ensiemanalises het nie ooreengestem met dié wat waargeneem is in die *in vitro* analises nie. Daarom moet verskillende interpretasies gegee word aan ensiemaktiwiteit en *in vitro* verteerbaarheidswaardes en die tyd van rumenvloeistof versameling moet altyd aangedui word wanneer rumenvloeistof gebruik word in navorsing of vir kommersiële doeleindes.

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LIST OF ABBREVIATIONS

α-AF	α-L-Arabinofuranosidases
CMC	Carboxymethylcellulose
CP	Crude protein
DNS	Dinitrosalicylic acid
HG	Homogalacturonan
iNDF	Indigestible neutral detergent fibre
ivSd	*In vitro* starch digestibility
N	Nitrogen
Nd	Nitrogen digestibility
NEFA	Non-esterified fatty acids
NDF	Neutral detergent fibre
NDFd	Neutral detergent fibre digestibility
RG I	Rhamnogalacturonan I
RG II	Rhamnogalacturonan II
RDP	Rumen degradable protein
RUP	Rumen ungradable protein
SCN	Suprachiasmatic nucleus
TMR	Total mixed ration
VFA	Volatile fatty acid

CHAPTER 1

GENERAL INTRODUCTION

In vitro systems using rumen fluid are the oldest, and still the most common laboratory methods in use for estimating digestibility in animal feeds. Using rumen fluid in fermentative *in vitro* systems has the potential to measure the ruminal *in vivo* rates of digestibility and fermentation patterns (Craig *et al*., 1984), assuming the only limiting factor during fermentation is the substrate itself. Relatively little attention has been paid to the effect of the handling of the liquor once harvested Considering that the microflora in the rumen, and respective enzymes, in the rumen fluid are the essential reagent of an *in vitro* assay, this should not be underestimated, especially because of the assumption that enzymatic activity is not limiting within the artificial micro-environment created. Analysing the enzymatic activity of rumen fluid in correlation with *in vitro* digestibility studies can provide a different perspective to the changes observed in ruminal starch, neutral detergent fibre (NDF) and protein digestibility. However, performing enzymatic activity studies parallel to *in vitro* digestibility studies have been hindered by the lack of a standard method of analysis and by the lack of knowledge surrounding the variables that could potentially influence the enzymatic activity. It is known that the composition of the ruminal microbial population is responsive to changes in various natural and feed related factors. However, the dynamic between the microbial population and enzymatic activity within the rumen, and how the enzymatic activity varies in response to various factors, are not well defined. This is especially true when referring to methodological differences used to extract enzymes from ruminal particulate matter for enzymatic analysis and the effect of the time of rumen fluid sampling on the enzymatic activity.

Traditionally, rumen fluid is used excluding the particulate matter. However, a higher concentration of rumen microbes is associated with the whole particulate fraction compared to the liquid fraction of the rumen content (Forsberg and Lam, 1977; Craig *et al*., 1987a). Microorganisms attached to feed particles tend to be retained in the rumen longer than the fluid, allowing for an

increased reaction time between the substrates and enzymes (Wang and McAllister, 2002). Therefore, microorganisms associated with the particulate fraction play a bigger role in the digestion of feed in the rumen (McAllister *et al*., 1994). The rumen inocula for *in vitro* digestibility studies are usually prepared by straining through cheesecloth layers. However, according to some authors (Craig *et al*., 1987b), blending of rumen fluid before straining through cheesecloth can increase the concentration of bacteria in the liquid fraction of the rumen contents by liberating some of the bacteria attached to the particles. Sonication and lysozyme treatment are other methods that have been used to free enzymes associated with the particulate fraction of rumen fluid. However, because of cell lysis caused by both sonication and lysozyme treatment, these treatment methods result in rumen fluid not utilisable for digestibility determination but solely for the determination of enzymatic activities. Additionally, lysozyme treatment is a laborious process, and the carbon tetrachloride needed for the treatment is not always easily obtainable. Therefore, blending and sonication are more practical enzyme extraction methods. Sonication has been used for the extraction of enzymes from rumen content, either alone or with lysozyme treatment (Patra *et al*., 2006, 2010; Rey *et al*., 2012). However, how the enzymatic activity of sonicated rumen fluid compares to the activity of untreated and blended rumen fluid has not been determined.

In addition to the different methods employed in the literature for the extraction of enzymes for enzymatic analysis, rumen fluid sampling time could also introduce another variable. When referring to cows fed a total mixed ration, the time of day (relative to the last feeding) that rumen fluid is sampled for analysis could be an area of variation for both *in vitro* and enzymatic analysis. When the time of rumen fluid sampling is not taken into consideration no allowance is made for variations caused by diurnal patterns and the effect it might have on starch, NDF and protein digestibility.

Numerous physiological activities in cows have been reported in the literature (Lefcourt *et al*., 1999; Piccione *et al*., 2007; Giannetto and Piccione, 2009) to follow a circadian pattern, including glucose, urea, non-esterified fatty acids (NEFA), total lipids, total cholesterol, haemoglobin,

creatinine, magnesium, phosphorus, respiratory rate and rectal temperature. Milk production in dairy cows has also been shown to follow a circadian pattern that is sensitive to the timing of feed intake (Rottman *et al*., 2014). The feeding time of dairy cows have the ability to retrain the daily rhythm of feeding, core body temperature, volatile fatty acids (VFA), ammonia, faecal NDF and indigestible neutral detergent fibre (iNDF) concentrations, lying behaviour, plasma blood urea nitrogen, insulin and glucose concentrations (Blackburn and Hobson, 1960; Russell *et al*., 1981; Niu *et al*., 2014). Cone *et al*. (1989) and Fickett and Allen (2002) showed that the extent of *in vitro* starch digestibility was also affected by feeding time. Fickett and Allen (2002) attributed the variation in digestibility rates on the difference in enzymatic activity of rumen fluid collected before and after feeding. There is, therefore, a reason to speculate if diurnal patterns for enzymatic activity might not also exist as bacterial numbers have already been shown to be influenced by the time of feeding (Bryant and Robinson, 1968).

The aim of this study was to better understand the complexity of rumen fluid in terms of enzymatic activity and, to identify how methodological differences in sampling and handling affect the enzymatic activity and in turn determine how this might affect *in vitro* digestibility measurements. The objective of the first study was to determine if blending or sonication could increase the amylolytic, fibrolytic and proteolytic activity of rumen fluid and to determine the effect of blending rumen fluid on the *in vitro* digestibility of starch, NDF and protein. Secondly, an objective to investigate if diurnal patterns in starch, NDF and protein digestibility and amylolytic, fibrolytic and proteolytic activity, respectively, exist in dairy cows, was set.

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CHAPTER 2

ENZYMATIC ACTIVITY OF RUMEN FLUID IN RUMINANTS: A REVIEW

2.1 INTRODUCTION

The rumen is a slightly acidic (pH 5.5-7.0) and anaerobic environment, with a temperature of 39-40°C. These conditions, together with the constant supply of food and ongoing elimination of fermentation products and food residues, creates a favourable environment for dense, diverse populations of rumen microflora to develop (Hungate, 1966). The microbial community contributes to the host animal's nutrient uptake by digesting feed particles, producing microbial vitamins, proteins and volatile fatty acids (VFA; Li *et al*., 2009). The ruminal microbial community includes bacteria (over 50 genera), ciliate protozoa (25 genera) and lower numbers of anaerobic fungi (5 genera) and bacteriophages (Gordon and Phillips, 1992; Hobson and Stewart, 1997; Morgavi *et al*., 2010). Bacteria are far more abundant in the rumen than fungi and protozoa and are responsible for most of the ruminal fermentative activity (Huntington, 1997; Dehority, 2003). However, modern DNA technology has allowed in the last years the isolation, characterization and identification of some of the ruminal microbes, contributing to our evolving understanding of the complex ruminal microbial ecosystem (Li *et al*., 2009; Morgavi *et al*., 2010). However it remains difficult to define the role played by each of the groups of ruminal microorganisms as the synergism and antagonism displayed by the different groups and even in the different genera within a group is complex and diverse (Hobson and Stewart, 1997). The methods used to study rumen microorganism can be divided into three categories namely, pure cultures, mixed culture *in vitro* fermentations and *in vivo* - *in vitro* procedures (Dehority, 2003).

In vitro systems using rumen fluid are the oldest, and still the most common laboratory methods in use for estimating digestibility in animal feeds. However, monitoring the rumen function of cows by analysing the enzymatic activity of rumen fluid in correlation with *in vitro* digestibility

studies can provide a different perspective to the changes observed in ruminal starch, neutral detergent fibre (NDF) and protein digestibility. Despite that, there is a limit on the number of published ruminal studies analysing endogenous enzymatic activities. Additionally, most of these studies pertained to the enzymatic activity of certain microbial isolates and not to the activity of the rumen fluid as a whole. Performing enzymatic activity studies parallel to *in vitro* digestibility studies is hindered by the lack of a standard method of analysis and by the lack of knowledge surrounding the variables that could potentially influence the enzymatic activity. It is known that the composition of the ruminal microbial population is responsive to changes in various natural and feed related factors (Hungate, 1966; Hobson and Stewart, 1997). However, the dynamic between the microbial population and enzymatic activity within the rumen, and how the enzymatic activity variates in response to various factors, are not well defined. The purpose of this review is to provide an overview of the key enzymes of microbial origin that play a role in starch, fibre and protein digestion in the bovine and ovine rumen and to highlight the main factors that influence their production and activity.

2.2 STARCH

Starch is often the main nutrient in the diet of high producing ruminants (Theurer, 1986). The main source of starch energy in ruminant diets is from grains, most often sorghum, maize and barley grain (Theurer, 1986; Huntington, 1997), containing 70 to 80% starch (Rooney and Pflugfelder, 1986; Nocek and Tamminga, 1991).

The two α-linked glucose polymers contained in starch are amylose and amylopectin (French, 1973; Chesson and Forsberg, 1997). Amylose, the simpler polymer, is an unbranched polymer of up to 6000 glucose units linked via α-1,4 bonds (French, 1973; El-Fallal *et al*., 2012). In a solution, amylose takes on a helical structure with each turn comprised of six glucose residues (Chesson and Forsberg, 1997). Amylose content within starch can range from anything between 0 to 80% and is dependent on the starch type and its genetic variation (Rooney and Pflugfelder, 1986). Amylose comprises 20 to 30% of normal cereal starches (Rooney and Pflugfelder, 1986). However, starches

with higher and lower amylose content exist, namely high-amylose maize that contains 40-80% amylose (Chesson and Forsberg, 1997) and waxy grains that contain minimal to no amylose (Rooney and Pflugfelder, 1986). Amylopectin is a larger polymer and constitutes 70 to 80% of normal starches (Rooney and Pflugfelder, 1986). It is a branched polymer of 10,000-100,000 D-glucose units linked via α-1,4 bonds with α-1,6 branch point occurring at every 20 to 25th glucose residue (French, 1973; Rooney and Pflugfelder, 1986; Kotarski *et al*., 1992).

Starch is deposited in granules within the endosperm (Svihus *et al*., 2005) and is typically divided into parts of unorganized amorphous areas and parts of extremely organized crystalline areas (French, 1973; Nocek and Tamminga, 1991). The crystalline areas are predominantly comprised of amylopectin (Rooney and Pflugfelder, 1986) with the branch points of amylopectin suspected to be located in the amorphous areas (Saibene and Seetharaman, 2010). The amorphous area is more sparse and abundant in amylose compared to the crystalline area (Rooney and Pflugfelder, 1986). Starch is intrinsically insoluble in water and, therefore, starch molecules gather together in plants to form starch granules (French, 1973). The size of these starch granules can be anything from less than 1-µm to more than 100-µm in diameter, being influenced by the type of plant tissue (French, 1973). The fact that starch is arranged into granules makes it moderately resistant to digestion by amylase (French, 1973). A list of the well-studied amylolytic enzymes, their mode of action and some of the ruminal microorganisms responsible for their production can be seen in Table 2.1.

2.2.1 Starch digestion

In ruminants, starch digestion commences in the rumen (Kotarski *et al*., 1992) as ruminants do not secrete salivary amylase (Mau *et al*., 2010; Ang *et al*., 2011) . Mammalian enzymes easily digest starch, but access is restricted because the granules are embedded in a protein matrix located in the seed endosperm (Kotarski *et al*., 1992). Therefore, ruminants are dependent on microbial enzyme production to aid in the digestion of unprocessed carbohydrates in the rumen. Amylolytic bacteria are mainly responsible for starch digestion in the rumen, with protozoa and fungi contributing

to a smaller degree (Huntington, 1997). *Fibrobacter succinogenes*, *Prevotella* (Previously *Bacteroides*) *ruminicola*, *Clostridium locheadii*, *Bacteroides succinogenes*, *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*, *Succinimonas amylolytica*, *Ruminobacter amylophilus* (previously *Bacteroides amylophilus)* and *Streptococcus bovis* are bacteria species that are able to utilise starch as a substrate in the rumen (Hungate, 1966; Dehority, 2003). For maximum digestion of starch, a combination of bacteria are required because not all bacteria produce the complete array of enzymes required for starch digestion (Huntington, 1997). Protozoa digest starch by engulfing the granules (Huntington, 1997). However, defaunation has been reported to improve ruminal starch digestibility and increase amylolytic activity (Mendoza *et al*., 1993). Fungi have the ability to penetrate the cuticle layer on cell surfaces consequently giving bacteria access to the fermentation sites (Akin and Rigsby, 1987; Ortega Cerrilla and Mendoza Martínez, 2003).

Amylolytic enzymes (collectively called amylases) commence their attack on starch on the surface of the molecule, spreading laterally to form conical pits. Starch granules are continually eroded by amylolytic action until they are completely dissolved (French, 1973). Amylases have the ability to digest the glycosidic linkages present in starches (El-Fallal *et al*., 2012). Amylolytic bacteria and fungi secrete amylases into the extracellular fraction of the rumen contents to carry out digestion (El-Fallal *et al*., 2012). These amylases are classified according to the way they attack glycosidic bonds and can be grouped into 4 types, namely (i) exoamylases (ii) endoamylases (iii) transferases and (iv) debranching enzymes (El-Fallal *et al*., 2012). The major amylases involved in starch hydrolysis are α-amylases, β-amylases, isoamylases, pullulanases and glucoamylases (Rooney and Pflugfelder, 1986).

2.2.2 Amylolytic activity

α-Amylase. Action from this enzyme causes a dramatic reduction in viscosity and the release of oligosaccharides from amylose (Chesson and Forsberg, 1997). Alphaamylase displays endo-amylase activity and hydrolyses starch molecules by randomly hydrolyzing α-1,4 glycosidic bonds

within starch, yielding maltose and linear and branched dextrins (Rooney and Pflugfelder, 1986). Different α-amylases differ in terms of the stage that random hydrolysis commences and declines, therefore each α-amylase is capable of producing a distinct group of oligosaccharides (Walker, 1965). Alpha-amylase is one of the enzymes that are readily involved in the hydrolysis of starch to glucose (French, 1973). The action of α-amylase on amylopectin leads to the production of maltotriose, maltose, limited amounts of glucose and mixed α-limit dextrins (Ortega Cerrilla and Mendoza Martínez, 2003). The action of α-amylase on amylose produces maltotriose, maltose and less frequently limited amounts of glucose (Ortega Cerrilla and Mendoza Martínez, 2003). The action of α-amylases and other debranching enzymes increases the yield of glucose from the digestion of starchy feeds because the branched and linear dextrins produced by these enzymes are more readily hydrolysed by intestinal glucoamylases (Rooney and Pflugfelder, 1986).

Ruminal bacteria (*B. fibrisolvens* 49, A38, D1; *S. ruminantium* HD4, D; *P. ruminicola* 23, B_14; *R. amylophilus* H18; *S. bovis* JB1) were grown in a medium containing starch, of these bacteria tested *R. amylophilus* H18, and *S. bovis* JB1 produced the highest amylase activity (Cotta, 1988). Cotta (1988) also found that the carbohydrate source in the growth medium influenced the growth rate of the ruminal bacteria, the quantities of amylose produced and the ratio of amylolytic activity between extracellular and cellular fractions. *B. fibrisolvens* 49 and A38, *S. bovis* JB1, *P. ruminicola* 23 and B_14 produced amylase when grown in a starch or maltose growth medium. However, amylase production was considerably less when grown in a glucose medium (Cotta, 1988). *S. bovis* JB1 and *B. fibrisolvens* 49 produced mainly cell-associated amylase when grown on starch. However, when grown on maltose, the amylase activity was almost entirely associated with the extracellular fraction. The extracellular amylolytic activity of these strains displayed endo-splitting activity when digesting amylose similar to that of α-amylase (Cotta, 1988).

The α-amylase produced by the ruminal anaerobic fungus *Neocallimastix frontalis* PN-1, grown on starch, was predominantly released into the culture medium with a small fraction (less than 20%) being associated with the fungal rhizoid (Mountfort and Asher, 1988). Sonication was unable

to increase the activity of the fungal tissue (Mountfort and Asher, 1988). The α-amylase produced by the fungus displayed endohydrolytic activity. The main products of starch digestion by the fungal α-amylase was longer-chain oligosaccharides, maltopentaose, maltotetraose, maltotriose and maltose (Mountfort and Asher, 1988).

The protozoa, *Eudiplodinium maggii* and *Diploplastron affine* isolated from the rumen of sheep, have also been identified as producers of α-amylase (Bełżecki *et al*., 2007; Wereszka, 2012).

β-amylase. This enzyme liberates maltose units in a successive fashion from the non-reducing side of the amylose chain by hydrolysing α-1,4-glycosidic linkages (Chesson and Forsberg, 1997; Min Ha *et al*., 2001). β-Amylase is unable to digest amylose in the presence of α-1-6 side chains linked to in-chain glucose units (Chesson and Forsberg, 1997). Additionally, β-amylase is only capable of hydrolysing 50% of amylopectin as the α-1,6-linked branch points hinder further digestion. Complete digestion of amylopectin and branched amylose requires the action of debranching enzymes, namely glucoamylase, isoamylase and pullulanases (Chesson and Forsberg, 1997).

Isoamylases. Isoamylases are debranching enzymes and hydrolyse α-1,6 glycosidic bonds inside starch molecules, yielding linear dextrins (Rooney and Pflugfelder, 1986). These enzymes solely hydrolyse the α-1,6 glycosidic bonds in amylopectin (Van Der Maarel *et al*., 2002; El-Fallal *et al*., 2012).

Pullulanases. Pullulanases are debranching enzymes and hydrolyse α-1,6 glycosidic bonds in amylopectin and pullulan molecules, yielding linear dextrins (Rooney and Pflugfelder, 1986; Van Der Maarel *et al*., 2002). The ruminal bacterium *R. amylophilus* 70 displayed pullulanase activity in maltose grown cells (Anderson, 1995). The polysaccharides activity of *R. amylophilus* 70 gave the impression of being cell associated, specifically with the soluble and membrane cell fractions (Anderson, 1995). However, the extracellular activities detected in this study could have been the result of cell lysis by sonication (Anderson, 1995).

α-Glucosidase. α-Glucosidase digests glucose into maltose and maltodextrins with low molecular weights (Chesson and Forsberg, 1997). *Lachnobacterium bovis*, strain YZ 87T and YZ 63,

was isolated from the rumen of a steer fed a 50% chopped alfalfa and 50% rolled barley diet *ad libitum* (Whitford *et al*., 2001). Both strains displayed α-glucosidase activity.

Maltase. Maltase converts the soluble oligosaccharides and dextrins, released during the breakdown of starch, into glucose (French, 1973). Bailey and Howard (1963) studied the properties of maltase produced by three ruminal protozoa namely, *Entodinium caudatum*, *Epidinium ecaudatum* and *Dasytricha ruminantium*. The maltase produced by *E. caudatum* displayed maximum activity at pH5.7-6.1 and 38°C (Bailey and Howard, 1963a). Both *E. caudatum* and *E. ecaudatum* displayed transferase properties (glucose to C-4 of maltose) when incubated with 1% (w/v) or higher levels of maltose concentration (Bailey and Howard, 1963a). Similarly, *D. ruminantium* displayed transferase properties (glucose to C- and C-6 of maltose) when incubated with 1% (w/v) or higher levels of maltose concentration (Bailey and Howard, 1963a) The maltase produced by *E. caudatum* displayed the highest activity at pH 6.7 and temperature of 38°C (Bailey and Howard, 1963a). The maltase produced by *D. ruminantium* displayed the highest activity at pH 5.5 (Bailey and Howard, 1963a). The protozoan, *D. affine*, isolated from the ovine rumen has also been identified as a producer of maltase (Wereszka, 2012).

Amyloglucosidase. Amyloglucosidase (also known as glucoamylase) is a debranching enzyme (Chesson and Forsberg, 1997) that displays exo-amylase activity by attacking terminal glucose residues of amylopectin or amylase (Rooney and Pflugfelder, 1986; El-Fallal *et al*., 2012) and subsequently produces β-limit dextrin and maltose or glucose (Rooney and Pflugfelder, 1986; El-Fallal *et al*., 2012). *N. frontalis* isolated from the bovine rumen displayed amyloglucosidase activity, as glucose was the only end product of starch digestion (Pearce and Bauchop, 1985). Additionally, the amyloglucosidase activity of *N. frontalis* was thirteen times higher in the extracellular fraction than in the insoluble fraction.

Amylopectinase. Amylopectinase targets α-1,6 linkages, debranching amylopectin to yield amylose (Chudzik, 2007; Hoffman, 2009). Amylopectinase activity produced by *R. amylophilus* 70 was measured by a reducing sugar assay with amylopectin as substrate (Anderson, 1995).

Amylopectinase was present in both the extra- and intracellular fractions of the bacterium. However, the extracellular activities detected in this study could have been the result of cell lysis by sonication (Anderson, 1995).

2.2.3 Factors interacting with ruminal starch digestion

Depression of starch digestion is usually the result of extraneous factors, for example, the presence of anti-nutrients, the presence of undamaged cell walls that make it impossible for enzymes to access the easily fermentable starches and, less frequently, the organisation and structure of the starch molecule itself (Chesson and Forsberg, 1997). Additionally, starch-protein interactions, grain vitreousness and type can also have an effect on the feeding value and digestibility of cereal starches (Thorne *et al*., 1983; Rooney and Pflugfelder, 1986; Allen, 2015).

Structure. The ratio of amylopectin to amylose will influence the digestibility of starch because amylopectin is more susceptible to the action of α-amylase (Ankrah *et al*., 1999). As mentioned earlier, amylose is a much smaller molecule than amylopectin. Therefore, the surface area of amylopectin is much larger than the surface area of amylose per molecule (Thorne *et al*., 1983). Additionally, amylopectin has numerous branched chains of glucose making its glucose chains more available to amylolytic attack than the glucose chains of amylose starch that are more strongly attached to each other by hydrogen bonds. Thorne *et al*. (1983) suggested that the difference in surface area between amylose and amylopectin might be the reason for amylopectin's higher rate of digestibility. Rooney and Pflugfelder (1986) suggested that the primary structural component of starch granules is amylopectin. The precise function of amylose in the granule has not been determined, however when waxy starches are warmed in water they display greater swelling (Rooney and Pflugfelder, 1986), they contain solely amylopectin and display faster rates of *in vivo* and *in vitro* digestion in comparison to non-waxy starches . This can suggest that amylose plays a role in limiting the swelling of starch granules (Rooney and Pflugfelder, 1986). Both the processing properties and digestibility of grains are affected by faint differences in the structure of starch granules (Rooney and

Pflugfelder, 1986). In general starch digestibility is inversely related to the amount of amylose present (Rooney and Pflugfelder, 1986). Waxy cereal starches (starches containing high levels of amylopectin) are some of the most digestible starch sources (Rooney and Pflugfelder, 1986).

Matrix interactions. Both protein and lipid interactions with starch can negatively affect starch digestibility. The interaction of both modified and native starches with protein can lower their susceptibility to enzyme hydrolysis as starch granules can be fully lodged in a protein matrix of the cereal grain endosperms (Rooney and Pflugfelder, 1986; Nocek and Tamminga, 1991). Starch digestibility is negatively associated with the storage proteins known as Prolamins (Gómez *et al*., 2016). Lipid-starch interactions can also decrease starch digestibility by limiting the accessibility of enzymes to the substrate (Svihus *et al*., 2005). Additionally, amylose-lipid content appears to negatively affect starch hydration and swelling (Vasanthan and Bhatty, 1996). The main phospholipid present on the surface of starch granules has been identified as phosphatidylcholine (Baldwin *et al*., 1997). Lysophosphatidylcholine, also identified on the granule surface, contains linolenic and palmitic fatty acids specifically (Baldwin *et al*., 1997).

Vitreousness. Increased vitreousness of maize kernels has been negatively correlated to ruminal *in situ* starch digestibility (Correa *et al*., 2002). Additionally, Fickett and Allen (2002) determined that the rate of *in vitro* digestibility was higher for floury endosperm than for vitreous endosperm (43.6 vs 28.5 %/h). In vitreous endosperm, starch digestibility is limited due to the relationship between protein and starch (Corona *et al*., 2006). These starch granules are lodged in a protein matrix, limiting access to substrates by hydrolytic enzymes.

Grain type. The starches found in wheat, oats and barley have a higher effective digestibility compared to the starches present in sorghum and maize (Gómez *et al*., 2016). Cooking of starches can improve their digestibility (Rooney and Pflugfelder, 1986). However, some starch sources like high amylose maize remain poorly digestible even after cooking (Rooney and Pflugfelder, 1986).

pH. The pH is one of the most variable factors within the rumen (Hungate, 1966), with fluctuations arising with feeding, frequency of feeding and as a result of the nature of the feed

(Dehority, 2003). The pH of the rumen is kept fairly constant by the secretion of alkaline saliva that is capable of neutralising, to an extent, the acid formed during microbial cell production (Hungate, 1966). However, too swift rates of fermentation and high levels of feed intake can cause increased production of acids by the ruminal microorganisms (Kotarski *et al.*, 1992). For ruminants, on what is termed a "normal" diet, the outer limit in pH lies between 4.5 and 7.5 and is usually attained 2 to 6-hours after once a day feeding (Dehority, 2003). Ruminal pH tends to decrease in an almost linear fashion to an increase in percentage maize in the diet (Dehority, 2003). The α-amylase (Cotta, 1988) and α-galactosidase (Bailey, 1963) producing bacterium, *S. bovis*, is an acid tolerant species (Russell and Dombrowski, 1980; Therion *et al*., 1982) that is able to grow at pHs 6.7 to 4.5 (Russell, 1991). *S. bovis* has the uncommon ability to allow its intracellular pH to decrease as the extracellular pH decrease, maintaining a near constant pH gradient between the intra- and extracellular environments (Russell, 1991).

2.3 FIBRE DIGESTION

Generally, the fibre portion or cell walls of plants are made up of 30-40% hemicellulose and pectin, 20% lignin and 15-40% cellulose (Doi and Kosugi, 2004). Herbivore enzymes are not capable of digesting plant wall polysaccharides (Flint *et al*., 2008). All microorganism groups present in the rumen, bacteria, fungi and protozoa, play a role in fibre digestion (Dijkstra and Tamminga, 1995). Digestion of these plant fibre materials yield oligomers which are hydrolysed into pentose, glucose and small carbon compounds (Doi and Kosugi, 2004). These carbon compounds, in turn, get converted to carbon dioxide (Doi and Kosugi, 2004). A list of the well-studied fibrolytic enzymes, their mode of action and some of the ruminal microorganisms responsible for their production can be seen in Table 2.1**.**

Cellulose. Cellulose is the main building block of plant biomass (Lynd *et al*., 2002) and is located in the cell wall of plants, particularly in the parts that add structural security (O'Sullivan, 1997). Unbranched cellulose is comprised of linear chains of anhydro-D-glucopyranose units linked

by β-1,4 bonds and are linked together by van der Waals' forces and hydrogen bonds (Orpin, 1984). Cellulose can consist of up to 10,000 linked units (Aspinall, 1980). Native cellulose is for the most part comprised of crystalline fibres with hydrogen binding together the individual molecules (Beguin, 1990). The degree of crystallinity varies between species, ranging from 32% in pine-tree pulp and 82% in cotton fibres (Orpin, 1984). Cotton fibres consist almost entirely of pure cellulose with both amorphous and crystalline regions (Beveridge and Richards, 1975). Beveridge and Richards (1975) showed that there was no significant difference in the rates of digestion for the crystalline and amorphous regions. However, cellulose fibres are rooted in a matrix of lignin and hemicellulose that restricts the access of cellulolytic enzymes (Beguin, 1990). More of the effect of lignin on digestion will be discussed later in the chapter.

The symbiotic relationship with cellulolytic microorganisms in the rumen is essential for cellulose utilisation (Russell and Wilson, 1996). Bacteria is the biggest digester of cellulose in the rumen (Russell and Wilson, 1996). The major cellulose-digesting bacteria identified in the rumen include cocci shaped *Ruminococcus flavefaciens* (Jindou *et al*., 2008), *Ruminococcus albus* and rod-shaped *F. succinogenes* (Krause *et al*., 2003), *B. fibrisolvens*, *B. succinogenes*, *Cellulomonos fimi* and the spore-forming rods *C. locheadii* and *Clostridium longisporum* (Hungate, 1966; Bayer *et al*., 1985). In addition to bacteria, fungi capable of digesting cellulose in ruminants include *Trichoderma reesei* (Bayer *et al*., 1985) and a group of fungi known as the anaerobic *Chytridiomycetes* (Lynd *et al*., 2002). *Neocallimastix* sp. is one of the most studied fibrolytic fungi's and belongs to the *Chytridiomycetes* group (Krause *et al*., 2003). Protozoa also have the capacity for cellulose digestion in the rumen, although its role is still poorly understood (Krause *et al*., 2003).

Hemicellulose. Hemicellulose is a combination of hetero- and homoglycans with the ratio depending on the plant species (Orpin, 1984) and is more concentrated in the secondary plant cell wall than the primary wall (Caffall and Mohnen, 2009). The main hemicelluloses are glucomannans, mannans, arabinogalactans, xylans, xyloglucans and arabinoxylans (Orpin, 1984). Xyloglucans are the main hemicelluloses of primary cell walls in dicots and are made up of β-1,4-linked glucan chains

with arabinose, xylose and xylosylgalactosylfucoside side-chains (Orpin, 1984). The main hemicellulose of monocots is β-1,4-xylan and secondarily xyloglucans (Orpin, 1984). Mannans display a three-dimensional structure similar to that of cellulose (Caffall and Mohnen, 2009). Xylan is made up of a repeated β-1,4-xylose backbone with branches of 4-O-methyl glucuronyl, arabinofuranosyl and acetyl groups (Moon *et al*., 2011). Beveridge and Richards (1975) showed that digestion-resistant hemicelluloses are protected by lignin and are otherwise chemically similar to digestible hemicelluloses. Xylan can be crosslinked to lignin by aromatic ester linkages (Moon *et al*., 2011).

Pectic substances. Pectic polysaccharides play an important role in the primary cell walls of gymnosperms and dicotyledons (O'Neill *et al*., 1990). Pectins are generally associated with the cell wall, but not covalently linked to it and are therefore almost totally digestible in the rumen (Nocek and Tamminga, 1991). The primary cell wall plays a valuable role in cell adhesion, structural robustness and signal transduction (Caffall and Mohnen, 2009). The pectic network in primary cell walls are comprised of homogalacturonan (HG) and rhamnogalacturonan I (RG-I) and rhamnogalacturonan II (RG-II; Albersheim *et al*., 1996). HG is a linear polymer of 1,4 linked α-D-galactopyranosyluronic acid (Ridley *et al*., 2001). RG-I consists out of a backbone of repeated disaccharides of alternating α-L-Rhamnose and α-D-GalA residues (Lerouge *et al*., 1993; Ridley *et al*., 2001). RG-II consists of a backbone of repeated 1,4 linked α-D-galactopyranosyluronic acid residues (Ridley *et al*., 2001). Covalent linkages exist between RG-I and RG-II and the backbone of HG (Caffall and Mohnen, 2009). Pectic polysaccharides polymers contain 1,4-linked α-D-galactosyluronic acid residues (O'Neill *et al*., 1990). The total dry matter pectin content in forage varies from 7.9% in latino clover, 1.5% in bromegrass and ranges between 4.7%-6.7% in five lucerne varieties (Pressey *et al*., 1963).

The enzymes that play a role in pectin digestion in the rumen include pectinesterases and polygalacturonases (Wright, 1960). These enzymes can be classified into two enzyme groups namely, hydrolases and lyases (Wojciechowicz and Tomerska, 1971). The hydrolase enzyme EC 3.2.15

degrades pectin into oligogalacturonides and eventually into galacturonic acid (Wojciechowicz and Tomerska, 1971). The lyase enzyme EC 4.2.99.3 degrades pectin into altered oligogalacturonides (Wojciechowicz and Tomerska, 1971).

Oligotrich and holotrich protozoa are capable of producing the pectin hydrolysing enzymes polygalacturonases and pectinesterases (Wright, 1960). Wright (1960) suggested that protozoa were unlikely solely responsible for pectin hydrolysis in the rumen. This was supported by *in vitro* studies (Howard, 1961; Dehority, 1969; Wojciechowicz and Tomerska, 1971). Using pure pectin as a medium, and donor cows solely fed on lucerne hay that is high in pectin, *P. ruminicola, B. fibrisolvens* and *Lachnospira multiparus* were identified as producers of pectin hydrolysing enzymes (Dehority, 1969). In a study by Wojciechowicz and Tomerska (1971) pectinesterase activity was detected by spectrophotometry for strains 1 and 52 of *P. ruminicola*.

2.3.1 Fibrolytic enzymes and factors interacting with their activity

Exo- and endocellulase, β-glucosidase, endoxylanase, β-xylosidase, α-amylase, licheninase, mannanase and acetyl xylan esterase are all glycosyl hydrolase enzymes produced by carbohydrate digesting microorganisms in the rumen (Krause *et al*., 2003). Microorganisms capable of digesting cellulose do so by producing exo- and endoglucanases that work together to hydrolyse cellulose (Bayer *et al*., 1985). Both endo- and exoglucanases are required for the degradation of crystalline cellulose (Orpin, 1984). Amorphous cellulose can be degraded by either endo- or exoglucanases (Orpin, 1984). Endoglucanases act on cellulose chains at the internal amorphous areas to produce cello-oligosaccharides of various lengths (Chesson, 1981; Orpin, 1984; Lynd *et al*., 2002; Doi and Kosugi, 2004). Exoglucanase attacks cellulose from either the non-reducing or the reducing end of the chain to produce cellobiose or glucose (Orpin, 1984; Lynd *et al*., 2002). Beta-glucosidase then digests the soluble cellobiose and cellodextrins to glucose (Lynd *et al*., 2002). The cellulases, endo-1,4-β-glucanase (endoglucanase), exo-1,4-β-glucanase (cellobiohydrolase and cellodextrinase), β-glucosidase (cellobiase) are all synergistically involved in the hydrolysis of cellulose, according to

the currently accepted model developed based on cellulolytic fungal studies (Wood, 1985; Huang and Forsberg, 1987).

Undefined endoglucanases. The anaerobic ruminal fungi, *F. succinogenes* S85, produced an endoglucanase enzyme that was mostly associated with the culture medium (Bae *et al*., 1993).

Endoglucanase of fungal origin targets β-glycosidic bonds randomly within cellulose molecules (Beguin, 1990). Endoglucanase also attacks swollen phosphoric acid cellulose and carboxymethylcellulose (CMC) in a nonspecific manner causing a dramatic decrease in chain length and to the production of cello-oligosaccharides (Chesson, 1981). *Orpinomyces sp.* strain PC-2 encodes for two enzymes, CelB and CelE, that displayed endoglucanase activity (Chen *et al*., 1998). CelB is a family 5 hydrolase enzyme (Chen *et al*., 1998). Both enzymes readily digested lichenin, CMC and barley β-glucan (Chen *et al*., 1998). CelE displayed partial activity towards ρNP-β-D-cellobiose and cellotriose (Chen *et al*., 1998). Neither could digest cellobiose. The end product of CMC, after hydrolysis by CelB and CelE, was glucose, cellotriose and cellobiose (Chen *et al*., 1998).

Strain SG4 of the anaerobic fungi *Orpinomyces joyonii,* isolated from the ovine rumen, produced two cellulases, CelB2 and CelB29. These enzymes were of the family 5 glycosyl hydrolases and most closely resembled the endoglucanases of *Neocallimastix patriciarum* (Qiu *et al*., 2000). These enzymes displayed an affinity towards long- and short-chain β-1,4-glucans (Qiu *et al*., 2000). The pH optimum for CelB2 and CelB29 was 6.6 and 5.8, respectively (Qiu *et al*., 2000). Enzymatic activity was determined spectrophotometrically, and a single unit of enzymatic activity was expressed as the amount of 1 nmol of ρ-nitrophenol liberated per minute (Qiu *et al*., 2000).

β-Glucanase. *N. patriciarum* expresses a β-glucanase, encoded by the *celB* gene (Zhou *et al*., 1994). It was mainly active against β-glucan but also displayed notable activity against cellulo-oligosaccharides, CMC and soluble xylan (Zhou *et al*., 1994). Xue *et al*. (1992) showed that the ruminal fungus, *N. patriciarum*, encodes for a polysaccharide hydrolase cDNA that contains three multifunctional catalytic domains, one encoding for high endoglucanase activity.

Endoglucanase. CMC has been widely used as a substrate in endoglucanase activity studies (Hungate, 1988). In a study by Mountfort and Asher (1985), endoglucanase activity was examined for PN-2, a strain of the fungi *N. frontalis*, isolated from the bovine rumen. Endoglucanase activity was measured based on the colourimetry method of Saddler and Khan (1980; Mountfort and Asher, 1985). The activity of endoglucanase was expressed as a single unit of endoglucanase equalling 1µg glucose equivalents liberated per minute (Mountfort and Asher, 1985). This study also found a reverse correlation between the enzyme activity and glucose concentration, with endoglucanase activity decreasing with an increase in glucose concentration. Mountfort and Asher (1985) suggested that glucose could play a role in the regulation of endoglucanase. An increase in the sugar concentration leads to a decrease in the enzyme activity, with endoglucanase activity being partially and totally inhibited at a concentration of 1.2 and 4 mg of glucose ml^{-1} respectively (Mountfort and Asher, 1985). The highest activity of the enzyme was observed when incubated with 2.5 mg of cellulose ml^{-1} (Mountfort and Asher, 1985). The study observed no increase in endoglucanase yield by shaking of the *N. frontalis* strain PN-2. The pH that corresponded to the highest activity for endoglucanase activity was observed at 6.0 (Mountfort and Asher, 1985). Similarly, the endoglucanase activity of bovine rumen fluid, analysed with two different forages, also displayed the highest activity at pH 6.0 (Farenzena *et al*., 2014). In a study by Huang and Forsberg (1987), 70% of the endoglucanase activity produced by *B. succinogenes* was associated with the cell-free supernatant fraction. However, Lee *et al*. (2002) reported that the CMC degrading enzyme, β-D-endoglucanase, present in the rumen of bulls on an alfalfa-based diet, was mainly bound to microbial cells. The problem with using CMC as a substrate, as shown by Fields *et al*. (1998), is that many non-cellulolytic organisms can degrade CMC by various β-glucan enzymes; therefore, running the risk of attributing cellulolytic activity to non-cellulolytic organisms.

Undefined exoglucanases. Exoglucanase activity, produced by the fungi *N. frontalis*, displayed low activity when using Sigmacell and Avicel as substrates (Mountfort and Asher, 1985). Even sonication was insufficient in improving exoglucanase activity, leading Mountfort and Asher

(1985) to the conclusion that the production of these two enzymes by the fungi was in low numbers and that cello-oligosaccharides can possibly serve as a better substrate for the measurement of exoglucanase activity by the ruminal fungi. Exoglucanase activity on Avicelose and Sigmacellose were measured based on the method by Ng *et al*. (1977). The culture was incubated with a citrate-phosphate buffer (pH 5.0) and substrate for 4-hours at 50˚C (Mountfort and Asher, 1985). The reaction was stopped with filtration, and dinitrosalicyclic acid was utilised for the determination of reducing sugars (Mountfort and Asher, 1985). The cellulase activity on Avicelose and Sigmacellose was expressed as an enzyme that liberated 1 µg glucose equivalents per minute (Mountfort and Asher, 1985).

Cellobiohydrolase. Cellobiohydrolase, also known as cellobiosidase, is an exoglucanase. Cellobiohydrolase of fungal origin, hydrolysis cellulose and cello-oligosaccharides by cleaving cellobiose units (in a stepwise fashion) from the non-reducing ends of the polysaccharide chain (Chesson, 1981; Beguin, 1990). Xue *et al*. (1992) showed that the ruminal fungus, *N. patriciarum*, encodes for a polysaccharide hydrolase cDNA that contains three multifunctional catalytic domains, one encoding for high cellobiohydrolase activity. *Orpinomyces* sp.Y102, isolated from the bovine rumen, encodes for CelC7 that displayed cellotriohydrolase and cellobiohydrolase activity (Chen *et al*., 2014). The optimum activity for cellobiohydrolase produced by *Orpinomyces* sp.Y102 was observed at pH 6.0 (Chen *et al*., 2014).

Cellodextrinase. *B. succinogene*s produces a cellodextrinase that is mainly located in the periplasmic space (Huang and Forsberg, 1987). The cellodextrinase displays an exo-like action, hydrolysing cellodextrins to glucose and cellobiose (Huang and Forsberg, 1987). Digestion of cellopentaose, cellotetraose and cellotriose, yielded cellobiose (Huang and Forsberg, 1987). However, the enzyme was unable to further digest cellobiose (Huang and Forsberg, 1987). The highest activity of the enzyme produced by *B. succinogene*s was observed at pH 5.9 and 6.2 (Huang and Forsberg, 1987). Around 50% of the activity was lost at pH 5.2 or 7.0 (Huang and Forsberg, 1987). *B. fibrisolvens* H17c, isolated from the bovine rumen, encodes for a gene *ced1* that displays

cellodextrinase activity and limited endoglucanase activity (Berger *et al*., 1990). This enzyme digests short-chain cellodextrins producing either cellobiose or cellobiose and glucose (Berger *et al*., 1990). Cellohexaose and cellotetraose were hydrolysed to cellobiose (Berger *et al*., 1990). Cellopentaose and cellotriose were hydrolysed to glucose and cellobiose (Berger *et al*., 1990). Although *ced1* displayed mainly cellodextrinase activity, it was able to degrade CMC partially. The endoglucanase inhibitor, methylcellulose, did not display an inhibitory effect towards *ced1*. Additionally, Berger *et al*. (1990) indicated that the *ced1* protein was absent of a substrate binding domain, suggesting that loose association of the enzyme was sufficient for hydrolysis.

β-glucosidase. Fungi originating β-glucosidase enzyme digests soluble cello-oligosaccharides and cellobiose to glucose (Chesson, 1981; Beguin, 1990). This is true for cello-oligosaccharides with a low degree of polymerisation (Chesson, 1981). The ruminal fungus, *N. frontalis*, has been identified as a producer of β-glucosidase. In a study by Mountfort and Asher (1985), with the use of glucose oxidase, cellobiase activity (a β-glucosidase) was determined by the amount of glucose released from cellobiose. Cellobiase activity was expressed as a single unit of enzyme liberating 1 µg glucose equivalents per minute (Mountfort and Asher, 1985). Cellobiase activity produced by *N. frontalis* was determined to be at its highest at pH 5.5 (Mountfort and Asher, 1985). Pearce and Bauchop (1985) observed greater cellobiose than glucose production from the digestion of Avicelose by *N. frontalis*, indicating that β-glucosidase probably played a bigger role in the digestion of cellulose in relation to cellulase. Moreover, β-glucosidase activity was 1.3 times higher in the extracellular fraction compared to the insoluble fraction (Pearce and Bauchop, 1985). In a study by Huang and Forsberg (1987), contrasting results were found where most of the cellobiase activity of *B. succinogene*s was cell bound.

In a study by Groleau and Forsberg (1981) β-1,4,-glucosidase-like activity was observed for *B. succinogenes* grown on either glucose or cellobiose. *L. bovis*, strain YZ 87^{T} and YZ 63, was isolated from the rumen of a steer fed a 50% chopped alfalfa and 50% rolled barley diet *ad libitum* (Whitford *et al*., 2001). Both strains displayed β-glucosidase activity.

α-Galactosidase. Alpha-galactosidase was present in the cell extracts of *S. bovis* cultured on glucose (Bailey, 1963). Melibiose and oligosaccharides containing α-(1→6)-linked galactose were readily degraded by the α-galactosidase (Bailey, 1963). However, manninotri-itol, melibi-itol and methyl α-D-galactosidase were only hydrolysed slowly (Bailey, 1963). The highest activity for α-galactosidase was observed at pH 5.6-6.3 (Bailey, 1963). Additionally, the enzyme can act as a transferase of tri-saccharide and tetra-saccharide to raffinose, sucrose and melibiose acceptors (Bailey, 1963).

L. bovis, strain YZ 87^T and YZ 63, was isolated from the rumen of a steer fed a 50% chopped lucerne and 50% rolled barley diet *ad libitum* (Whitford *et al*., 2001). API AN-IDENT strips were used to determine enzymatic activity. Both strains positively displayed α-galactosidase activity. Complete genome sequencing of the gram-negative bacterium, *F. succinogenes* S85, has revealed encoding for numerous amylases, including α-galactosidase (Jun *et al*., 2007).

Extracts of the protozoan, *E. ecaudatum*, contained α-galactosidase activity (Bailey and Howard, 1963b). The protozoan enzyme was able to hydrolyse galactosylgalactosyl-glycerol, melibiose, other α-(1→6)-linked galactose containing compounds and galactosylgalactose (Bailey and Howard, 1963b). The highest activity for this protozoan enzyme was observed at pH 5.0-5.5 (Bailey and Howard, 1963b). Additionally, the α-galactosidase can act as a transferase when either 2% galactosylgalactosyl-glycerol, galactose or melibiose is present (Bailey and Howard, 1963b).

Mannanase. Mannanase, or β-D-Mannanase, is an endo-acting hemicellulase (Suen, 2011) that catalysis the breakdown of β-D-1,4-mannopyranosyl linkages within the mannan-containing polysaccharides, such as galactoglucomannans, galactomannans and glucomannans (Stålbrand *et al*., 1993). Hydrolysis of mannan by mannanase releases short-chain β-1,4-manno-oligosaccharides (van Zyl *et al*., 2010). A complete genome sequence was performed on *F. succinogenes* S85 (Suen, 2011) and analysis of the sequences revealed encoding of a high number of carbohydrate-hydrolysing enzymes (Suen, 2011). Degradation and utilisation assays showed that even though *F. succinogenes* S85 was capable of degrading numerous polysaccharides, it could only make use of cellulose and

cellulose degradation products (Suen, 2011). This suggests that *F. succinogenes* S85 produces hemicellulases solely for the removal of hemicellulose, thereby improving cellulose accessibility (Suen, 2011). *F. succinogenes* S85 encodes for a number of endo-hemicellulases, including mannanase among others (Jun *et al*., 2007; Suen, 2011).

Lichenase. The ruminal bacterium, *B. succinogenes*, produces 1,3-1,4-β-D-glucanase (lichenase; Erfle *et al*., 1988). The activity of the enzyme was restricted to lichenin and oat β-D-glucan. Hydrolysis of lichenin by this enzyme mainly yielded a pentasaccharide and a trisaccharide at 9.5% and 82% respectively (Erfle *et al*., 1988). Hydrolysis of oat β-D-glucan by this enzyme mainly yielded a tetrasaccharide and a trisaccharide at 29.6% and 63.5% respectively (Erfle *et al*., 1988). The highest activity for this bacterial lichenase was observed at pH 6.0 (Erfle *et al*., 1988). As previously mentioned, the bacterium *F. succinogenes* S85 encodes for a number of endo-hemicellulases, including lichenase among others (Jun *et al*., 2007; Suen, 2011).

The ruminal fungi, *Orpinomyces* sp. strain PC-2, has also been identified as a producer of 1,3-1,4-β-D-glucanase (Chen *et al*., 1997). The lichenase from *Orpinomyces* sp. strain PC-2 is similar biochemically to 1,3-1,4-β-D-glucanase of bacterial origin (Chen *et al*., 1997). The activity of the fungal enzyme is restricted to barley β-glucan and lichenin (Chen *et al*., 1997). Similarly, hydrolysis of lichenin mainly yielded a pentasaccharide and a trisaccharide (Chen *et al*., 1997). The hydrolysis of β-glucan mainly yielded a tetrasaccharide and a trisaccharide (Chen *et al*., 1997).

Xylanases. Synergistic action of endoxylanases, β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetyl xylan esterases and feruloyl esterases are required for xylose depolymerization (Dodd and Cann, 2009). *Polyplastron multivesiculatum* was the first ruminal protozoa identified with the capacity to produce fibre degrading enzymes. The first xylanase identified belonged to the glycoside hydrolase 11 family (Devillard *et al*., 1999). This enzyme displayed the highest activity at pH 6.5 (Devillard *et al*., 1999). The second xylanase identified belongs to the glycoside hydrolase 10 family and is encoded by gene *xyn10B* (Devillard *et al*., 2003). This enzyme displayed the highest activity at pH 7.0 and 39˚C (Devillard *et al*., 2003). The ruminal fungus *N. frontalis* is able to utilise

xylan for growth *in vivo* (Orpin and Letcher, 1979). The fungus *N. fontalis* PN-1 releases most of its xylanases into the culture fluid. However, the portion associated with the fungal rhizoid increased with sonication and toluene-ethanol-Triton treatment (Mountfort and Asher, 1989). The xylanase produced by this fungus displayed endo-hydrolytic activity on the breakdown of xylan, producing mainly xylobiose and xylo-oligosaccharides in lesser quantities (Mountfort and Asher, 1989). Xylan made for a more effective substrate for the growth of xylanases compared to glucose, xylose and cellobiose (Mountfort and Asher, 1989). Additionally, optimum activity as observed at pH 5.5 (Mountfort and Asher, 1989). Xue *et al*. (1992) showed that the ruminal fungus, *N. patriciarum*, encodes for a polysaccharide hydrolase cDNA that contains three multifunctional catalytic domains, one encoding for high xylanase activity.

The bacterium *R. albus* has been shown to produce a cellulosome that contains at least 11 catalytic domains, displaying xylanase activity amongst others (Ohara, 2000). Complete genome sequencing of the bacterium *F. succinogenes* S85 was shown to encode for a number of hemicellulases, including various xylanases (Jun *et al*., 2007; Suen, 2011). Additionally, the xylanases produced by *B. fibrisolvens* displayed mostly extracellular fluid associated activity (Hespell *et al*., 1987). The xylanase activity of bovine rumen fluid, analysed with two different forages, displayed the highest activity at pH 6.0 (Farenzena *et al*., 2014).

Endoxylanase. Endoxylanases are responsible for the degradation of the xylan backbone (Lagaert *et al*., 2014), yielding xylooligosaccharides (Lee *et al*., 1993). *R. albus* 8 is known for being one of the most active fibers degrading ruminal bacteria (Moon *et al*., 2011). Partial genome sequencing of the ruminal bacterium *R. albus* 8, has revealed encoding for multiple endoxylanases as well as the accessory enzymes α-glucuronidase, α-L-arabinofuranosidases and β-xylosidase (Moon *et al*., 2011).

β-Xylosidase. β-Xylosidase acts on xylooligosaccharides to yield xylose (Lee and Forsberg, 1987). β-Xylosidase was isolated from the anaerobic fungus *N. fontalis* with the use of gel filtration and anion-exchange chromatography (Garcia-Campayo and Wood, 1993). This enzyme acted on

xylooligosaccharides and xylobiose (Garcia-Campayo and Wood, 1993). The highest activity for β-xylosidase from *N. fontalis* was observed at pH 6.4 (Garcia-Campayo and Wood, 1993). Additionally, the enzyme was inhibited by the end product (D-xylose) of its natural substrate xylooligosaccharides (Garcia-Campayo and Wood, 1993).

α-L-Arinofuranosidases. α-L-Arabinofuranosidases (α-AF) cleaves branched arabinoses from the xylan backbone (Lagaert *et al*., 2014). α-L-Arabinofuranosidases was found in the extracellular broth of *R. albus* 8 cultures (Greve *et al*., 1984). α-L-Arabinofuranosidases of *R. albus* 8 was capable of removing arabinosyl residues from alfalfa cell walls, hemicellulosic and pectic polysaccharides (Greve *et al*., 1984). The optimum activity for this enzyme was observed at pH 6.8 (Greve *et al*., 1984). α-L-Arabinofuranosidases was isolated from *B. fibrisolvens* GS113 (Hespell and O'Bryan, 1992). The enzyme acted on furanoside and methylumbelliferyl-a-L-arabinofuranoside (Hespell and O'Bryan, 1992). The highest activity for α-AF from *B. fibrisolvens* GS113 was observed at pH 6.0 to 6.5 (Hespell and O'Bryan, 1992).

α-Glucuronides. The role of α-glucuronides is to act on 4-O-methyl-D-glucuronic acid residue branches linked via α-1,2-glycosidic bonds to the main xylan chain (Biely *et al*., 2000). The anaerobic fungus, *Piromonas communis* isolated from the ovine rumen, has been shown to produce α-glucuronide activity when grown on cultures containing birchwood sawdust, birchwood xylan, barley straw or filter paper (Wood and Wilson, 1995). Glucoronide activity was determined by the amount of α-(4-O-Methyl)-D-glucuronidase released from a culture containing O-α-(4-O-methyl-D-glucopyranosyluronic acid)-(1→2)-D-xylopyranose and O-α-(4-O-methyl-D-glucopyranosyluronic acid (1→2)-O-β-D-xylopyranosyl-(1→4)-D-xylopyranose and expressed as one unit of activity equaling 1 μmol 4-O-methyl-D-glucopyranosyluronic acid/ min at 50°C (Wood and Wilson, 1995). The enzyme displayed the highest activity at pH 5.5 (Wood and Wilson, 1995).

Acetylxylan esterase. Acetylxylan esterase plays a role in the hydrolyzation of lignin-hemicellulose linkages of the forage cell wall (McDermid *et al*., 1990). They remove acetyl moieties from arabinoxylan by cleaving ester bonds (Blum *et al*., 1999). Action by these enzymes improves

access to cellulose in plant cell walls (McDermid *et al*., 1990). *Orpinomyces* sp. strain PC-2 encodes for an acetylxylan esterase gene (Blum *et al*., 1999). The enzyme of *Orpinomyces* sp. strain PC-2 displays the highest activity at pH 9.0 (Blum *et al*., 1999). Acetylxylan esterase was purified from the culture fluid of *F. succinogenes* S85 (McDermid *et al*., 1990). The enzyme acted on acetyl residues from birchwood acetylxylan (McDermid *et al*., 1990). The acetyl esterase of *F. succinogenes* S85 displayed the highest activity at pH 7.0 (McDermid *et al*., 1990). Additionally, *N. patriciarum* encodes for two genes that display acetylxylan esterase activity (Dalrymple *et al*., 1997).

Feruloyl esterase. Feruloyl esterase catalyses the hydrolysis of ester bonds between ferulic acid and polysaccharides (Wong, 2013). Feruloyl esterase was isolated from a microbial metagenome of bovine origin, and its gene was subsequently cloned into *Escherichia coli* (Wong, 2013). Characterization of the enzyme using natural substrates revealed that the enzyme only hydrolyses the ferulic acid ester linked xylan to arabinosyl residues and not on the ether-linked lignin and ferulic acid (Wong, 2013).

Pectinesterase. The hydrolase enzyme, pectinesterase EC 3.1.1.11, plays a role in the freeing of methyl ester groups to yield polygalacturonic acid (Wojciechowicz and Tomerska, 1971). Lineweaver and Jansen (1951) described pectinesterase as an enzyme that catalysis the cleaving of the ester linkages of pectic substances to produce methanol and pectinic or pectic acids. In a study by Wright (1960) pectinesterase activity of rumen protozoa was determined by using a manometric method to estimate the release of methanol by the enzyme. Pectinesterases displayed an increase in activity with an increase in pH up till pH 7.8 (Wright, 1960). Demethylation of pectinesterases occurs at pH 8.0 and above (Wright, 1960; Abou Akkada and Howard, 1961). Pectinesterase activity in the study by Abou Akkada and Howard (1961) was determined by incubating the enzyme, substrate and buffer together at 38˚C for 4-hours together with blanks containing (1) water and (2) water and methanol. The highest activity for pectinesterase produced by the mixed ruminal protozoa (*Isotricha intestinalis* containing *Isotricha prostoma* and *D. ruminantium*) was observed at pH 8.6 to 8.8 (Abou Akkada and Howard, 1961).

Polygalacturonase. Polygalacturonases are pectin depolarising enzymes and are involved in the cleaving of C-1-O bonds (Aspinall, 1980). Polygalacturonases are subdivided into endo- and exo-acting, depending on the site where attack commences (Wright, 1960). In the study by Wright (1960) rumen fluid was collected from a rumen-fistulated cow, and polygalacturonase activity of ruminal protozoa was determined by calculating the change in the relative viscosity of a pectin solution incubated in a water bath at 38˚C (Wright, 1960). The polygalacturonases displayed highest enzymatic activity between pH 6.5-7.2 (Wright, 1960). However, Abou Akkada and Howard (1961) reported that the polygalacturonases produced by the ruminal protozoa, mixed *I. intestinalis* containing *I. prostoma* and *D. ruminantium*, displayed the highest activity at around pH 8.7-9. There was also a difference obtained in the end product of pectin hydrolysis by polygalacturonase within these two studies. Wright (1960) reported the hydrolysis yielded mainly galacturonic acid with low oligouronides. However, Abou Akkada and Howard (1961) reported that the end product was mainly oligouronides with low galacturonic acid. They attributed the difference to the presence of pectolytic organisms in the protozoal suspension of the study performed by Wright (1960). Polygalacturonase activity in the study by Abou Akkada and Howard (1961) was determined by incubating polygalacturonic acid with a buffer and enzyme solution at 38˚C for 4-hours. The reducing sugars were determined by the photometric method of Nelson (1944) and was used to calculate the degree of hydrolysis of the substrate (Abou Akkada and Howard, 1961).

Kopečný and Hodrová (1995) showed that the H15 strain of the fungus *Neocallimastix sp.* isolated from the ovine rumen produced polygalacturonase in both the exo- and endocellular fractions. The polygalacturonase activity for the H15 strain was highest at pH 7.5 (Kopečný and Hodrová, 1995).

2.3.2 The role of microbial adhesion, cell-associated enzymes and cellulosomes in fibre digestion

Adhesion of microorganisms to plant cell walls is seen as the first critical step in the digestive process (Chesson, 1981) and places the enzymes within close proximity of the substrates in addition

to orientating the adhering cell in such a way as to promote substrate uptake (Forsberg *et al*., 1997). Microbial adhesion increases the time cell associated microorganisms are present in the rumen that would otherwise have been expelled by the normal flow of the liquid phase in the digestive tract (Chesson, 1981). The consequence is that a higher concentration of rumen bacteria is associated with the whole particulate fraction than there is with the liquid fraction of the rumen content (Hungate, 1966). It has also been proved that improved digestibility associated with the particulate fraction can be attributed to the attachment of bacteria to solid particles (Hungate, 1966). Forsberg and Lam (1977) found that approximately three quarters of ATP of microbial origin was associated with the particulate fraction of the rumen contents in a cow fed a hay diet. Craig *et al*. (1987) found that 70 to 80% of the microbial mass was associated with the particulate fraction. Cheng *et al*. (1983) found that more or less 70% of the bacteria present in the rumen was associated with the particle fraction. McAllister *et al*. (1994) categorised the particle associated population of the rumen into 3 categories: those associated with the liquid fraction; those loosely bound to particles and those firmly bound to particles. The liquid category was further divided into microorganisms detached from feed surfaces and microorganisms associated with the soluble feed constituents within the rumen fluid fraction (McAllister *et al*., 1994). The main route of microbial adhesion seems to be colonisation by epidermal lesions (Chesson, 1981). Microorganisms associated with the particulate fraction plays a bigger role in the digestion of feed in the rumen (McAllister *et al*., 1994). Beveridge and Richards (1975) suggested that the lag period of ± 10-hours, observed before the digestion of cotton commences *in vivo*, can be attributed to the time needed for the cellulolytic bacteria to attach to fibre particles and for the colonies to proliferate in the bovine rumen.

Bacteria are usually surrounded by extracellular structures, mostly fibrous polysaccharide glycocalyx (Cheng *et al*., 1977, 1981). These extracellular slime layers can assist in the adhesion of bacteria to surfaces (Cheng *et al*., 1977; Cheng *et al*., 1981), possibly also enclosing enzymes produced by bacteria in these structures. The cellobiase activity of *B. succinogenes*, grown on either glucose or cellobiose, was for the main part associated with the membrane of damaged cells, leading

to the conclusion that cellobiase is firmly cell-associated (Groleau and Forsberg, 1981). The only end product detected for cellobiase action on cellobiose was glucose (Groleau and Forsberg, 1981). Of the extracellular endoglucanase activity produced by *B. succinogenes*, 50-62% was related to the sedimentable membranous matter, supporting the hypothesis that the bacterium produced a cell associated cellulase (Groleau and Forsberg, 1981). This was supported by a study completed by Forsberg *et al*. (1981) who found that *B. succinogenes* grown on cellulose released more than 80% of its xylanase, aryl-β–xylosidase and endoglucanase and 50% of its aryl-β-glucosidase into the culture medium at 96-hours of incubation (Forsberg *et al*., 1981). After 168-hours of growth, ±50% of the enzymes in the culture medium was associated with the sedimentable subcellular membranous vesicles (Forsberg *et al*., 1981). These vesicles were observed free in the culture medium and as well as attached to cellulose (Forsberg *et al*., 1981). The vesicles produced by *B. succinogenes* contributed the cellulolytic and hemicellulolytic activity (Forsberg *et al*., 1981).

In a study by Williams and Strachan (1984) the activity level for glycoside hydrolases and polysaccharidases produced by protozoa and bacteria in the bovine rumen was analysed to determine their location of highest activity. These enzymes, responsible for cell wall degradation, displayed the highest activity in the particle-associated adherent population (Williams and Strachan, 1984). The particle associated subpopulation aids in the retention of microorganisms in the rumen and creates a favourable environment where organisms can maintain accessibility towards the substrates (Williams and Strachan, 1984).

Most anaerobic cellulolytic bacteria produce cellulosomes to aid in the adhesion and hydrolysis of cellulose (Bayer *et al*., 1985). Some anaerobic ruminal fungi also utilise cellulosomes in the digestion of insoluble polysaccharides. Cellulosomes are extracellular enzyme complexes, containing multiple carbohydrate-active enzymes and a scaffolding protein, capable of efficiently degrading cellulose and hemicellulose (Bayer *et al*., 1985; Doi and Kosugi, 2004). These multienzyme complexes display a particularly high activity against crystalline cellulose (Ohara, 2000). The greatest feature of the cellulosomes is the incorporation of carbohydrate-active enzymes

into the scaffolding protein that is controlled by the protein-protein interactions of dockerin and cohesion domains (Jindou *et al*., 2006). Each enzyme contains a dockerin domain that acts as a docking domain for enzymes that interact with cohesion domains (Bayer *et al*., 1994; Raghothama *et al*., 2001). The cohesion domain conducts the incorporation of the enzymes into the scaffolding protein (Bayer *et al*., 1994; Raghothama *et al*., 2001). Binding of cellulosomes to plant cell walls is made possible by carbohydrate-binding modules (Boraston *et al*., 2004). The interaction of type II cohesion (positioned at the cell envelope) and type II dockerin (positioned in the scaffolding) plays a role in the anchoring of cellulosomes to the plant cell wall (Fontes, 2010). Carbohydrate-binding modules are generally associated with glycoside hydrolases responsible for the degradation of insoluble polysaccharides (Boraston *et al*., 2004). These binding modules can either bind to components of the cell wall or insoluble polysaccharides like glycogen and starch (Boraston *et al*., 2004). A list of ruminal bacteria and fungi that digest cellulose by producing cellulosomes can be seen in Table 2.2.

Table 2.2 List of known anaerobic bacteria and fungi that utilise cellulosomes to digest insoluble polysaccharides.

Microorganism	**Reference**
Bacteria	
Clostridium cellobioparum	Lamed *et al*. (1987)
Ruminococcus albus	Lamed *et al*. (1987), Ohara (2000)
Ruminococcus flavefaciens	Jindou *et al*. (2006)., Jindou *et al*. (2008), Venditto *et al*. (2015)
Fungi	
Neocallimastix patriciarum	Zhou *et al*. (1994), Dalrymple *et al*. (1997)
Orpinomyces joyonii	Qiu *et al*. (2000)
Orpinomyces sp.* strain *PC-2	Chen *et al*. (1998), Steenbakkers *et al*. (2001)

In ruminococcus species, protuberance-like structures have been identified on their cell surface that is similar to the structures of *Clostridium thermocellum* that display cellulosome

association. Protuberance type structures have been shown to bind to cellulosomes in the sewage bacterium *C. thermocellum* (Bayer *et al*., 1985). It seems like the fibrolytic enzymes of *Ruminococcus* species are organised into similar cellulosome structures (Weimer *et al*., 2009). These cellulosomes aid in the adhesion of bacteria to insoluble substrates (Lamed *et al*., 1987). Similarly, the bacterium *Clostridium cellobioparum*, isolated from the bovine rumen (Hungate, 1944), positively displayed protuberance-type structures on its cell surface (Lamed *et al*., 1987).

R. albus, isolated from the bovine rumen (Sijpesteijn, 1949), has been identified as a cellulosome producing cellulolytic bacterium (Lamed *et al*., 1987). The cellulosome produced by *R. albus* contains at least 11 catalytic subunits that display xylanase or endoglucanase activities (Ohara, 2000). *R. albus* possesses at least two mechanisms that aids in the adhesion of the bacterium to cellulose (Miron *et al*., 2001). Namely, a CbpC (Pil)-protein mechanism and a cellulosome-type mechanism.

R. flavefaciens is a highly important cellulolytic bacterium in the rumen (Jindou *et al*., 2008) capable of utilising cellulosomes to digest cellulose (Jindou *et al*., 2006; Venditto *et al*., 2015). The first molecular evidence for this was the fact that xylanases and cellulases produced by *R. flavefaciens* 17 isolated from the bovine rumen encodes for dockerin-like-sequences (Kirby *et al*., 1997). The *R. flavefaciens* strain FD-1 encodes for a C-terminal dockerin and glycoside hydrolase enzyme (GH9) that is linked to two non-catalytic carbohydrate-binding modules (Venditto *et al*., 2015). Ding *et al*. (2001) identified two cellulosome-associated proteins, ScaA and ScaB in *R. flavefaciens*. The data suggested ScaA acted as a scaffolding-like protein of cellulosomes and ScaB served as a binding protein (Ding *et al*., 2001). The adhesion of *R. flavefaciens* to cellulose is made possible by two mechanisms, the carbohydrate epitopes of the glycocalyx layer and cellulosome-like complexes (Miron *et al*., 2001).

The *N. patriciarum*, isolated from the ovine rumen (Zhou *et al*., 1994), produces two enzymes, BnaA and BnaC that displayed acetylxylan esterase activity (Dalrymple *et al*., 1997). These enzymes are likely constituents of cellulosome complexes (Dalrymple *et al*., 1997). As previously mentioned,

O. joyonii produces two cellulases, CelB2 and CelB29. Qiu *et al*. (2000) suggested that they could be immobilised to the cellulosome, because *CelB2* and *CelB29* contained scaffold-binding sequences and lacked cellulose-binding domains.

The general opinion is that enzymes containing non-catalytic repeated peptide domains are fungal cellulosome subunits (Steenbakkers *et al*., 2001). The CelE enzyme produced by *Orpinomyces* sp. strain PC-2, isolated from the bovine rumen (Borneman *et al*., 1989), contains a non-catalytic repeated peptide domain suggesting that CelE forms part of the cellulosome produced by the fungi strain PC-2 (Chen *et al*., 1998).

Although cellulosomes aid greatly in the digestion of insoluble polysaccharides, not all cellulolytic anaerobes go about digesting substrates using cellulosomes (Ohara, 2000). Additionally gene-centric metagenomics of randomly sampled pyrosequence data from 1 pooled liquid microbiome sample and 3 fibre-adherent microbiomes harvested from the same bovine rumens revealed that initial colonisation of fibrous particles appears to be by the organisms producing enzymes that target the easily available side chains of complex polysaccharides and not the more resistant main chains (Brulc *et al*., 2009). Therefore, in contrast to previous evidence that stated the most important organisms responsible for plant cell wall hydrolysis were associated with the particulate fibrous fraction of the rumen content (McAllister *et al*., 1994; Forsberg *et al*., 1997), Brulc *et al*. (2009) suggested that plant cell wall hydrolysis is rather a dynamic process where the initial colonisation of organisms is later replaced by a another set of organisms responsible for degrading the main chains of xylan and cellulose. *F. succinogenes*, for one, does not hydrolyse cellulose with the use of cellulosomes (Suen, 2011). However, attachment of *F. succinogenes* is required for hydrolysis (Suen, 2011).

2.3.3 Factors interacting with ruminal fibre digestion

pH. The enzymatic activity of cellulolytic bacteria decreases dramatically with even the slightest decrease in ruminal pH because cellulolytic bacteria are not able to grow in a low pH

environment (Russell and Wilson, 1996). A low extracellular pH causes an increase in intracellular ions from VFA and an increase in the pH gradient causes anion toxicity in cellulolytic bacteria (Russell and Wilson, 1996). Mould *et al*. (1983) reported that cellulolytic enzymatic activity was completely inhibited when ruminal pH reached less than 6.0. This was supported by Hoover (1986) who showed a decreased growth rate or complete inhibition of fibrolytic microorganisms when the pH reached 5.5 or 5.0.

In an *in vitro* study by Stewart (1977) the effect of pH on cellulolytic activity was investigated. When the pH of the rumen fluid with added barley was allowed to fall, the rumen content displayed cotton-solubilising depression as well as depression in the filter paper-degrading bacterial counts (Stewart, 1977). The effect of pH on ruminal cellulolytic activity was examined by the addition of artificial saliva. A decrease in pH lead to a decrease in cellulolytic activity coinciding with a decrease in the filter paper-degrading bacterial count (Stewart, 1977).

Additionally, an *in vitro* study was conducted on bovine rumen fluid to determine the effect of pH on bacterial adhesion, enzyme activity and forage degradability using ryegrass and bermudagrass (Farenzena *et al*., 2014). Ryegrass displayed higher bacterial adhesion, dry matter digestibility and NDF digestibility and these results were positively and linearly affected by increased pH ($P < 0.05$). For both ryegrass and bermudagrass, there was a significant quadratic effect of pH on xylanase and endoglucanase activity ($P < 0.05$). In this study, there was a direct correlation between pH and bacterial adhesion and between pH and forage degradability. Similarly, Sung *et al*. (2007) has reported that maintaining a ruminal pH greater than 6 is essential for the efficiency of fibre digestion and microbial attachment.

Matrix interactions. The energy availability of high forage diets is limited due to the high concentration of forage cell walls and low digestibility of forage (Jung and Allen, 1995). In forages, 300 to 800 µg per g of dry matter is represented by the fibre (cell wall) portion (Hatfield *et al*., 1999). Fibre represents a large portion of the ruminant diet. However, less than half of it is readily digested (Hatfield *et al*., 1999). Therefore, cell walls play a critical role in discerning the quality of forage

(Hatfield *et al*., 1999). Structural proteins play a fundamental role in cross-linking cell wall constituents (Hatfield *et al*., 1999). Lignin is, however, indirectly the greatest inhibitor of cell wall degradation (Hatfield *et al*., 1999). Lignin is a polymer that interacts with other polymers in the cell wall to equip the wall with structural stability and in turn indirectly results in resistance to digestion and resistance against water permeation (Hatfield *et al*., 1999).

Hydroxycinnamate interactions are responsible for linking polysaccharides to lignin covalently (Hatfield *et al*., 1999). Ferulic acid bridges also play a role in linking lignin to cell wall polysaccharides and possibly play a role in limiting cell wall degradation (Jung and Allen, 1995). Lignin content can vary within cell walls depending on the development stage of the fibre, location in the cell wall and the presence of environmental stressors (Campbell and Sederoff, 1996). Lignin also displays variation in its quantity and organisation between cell types, between plant tissues within a specific plant and even within plants of the same specie (Campbell and Sederoff, 1996). For example, in the genus *Pinus*, the lignin content can range from 25% to 30% in *Pinus monticola* and *Pinus palustris*, respectively (Campbell and Sederoff, 1996).

Additionally, the degree of resistance directly relates to the degree of crystallinity within cellulose molecules (Chesson and Forsberg, 1997). Chesson (1981) reported the lowest rates of digestion were associated with high crystallinity-cellulose when incubated in the ovine rumen.

Tannins. Tannins are found in the cell vacuoles of some legumes such as birdsfoot trefoil and sainfoin (McAllister *et al*., 1994). They are polyphenols of plant origin and are of two types, namely hydrolysable tannins, condensed tannins or a combination of the two (McSweeney *et al*., 2001). Hydrolysable tannins are a polyester of gallic acid, and assorted individual sugars and condensed tannins are polymers of flavanoids (McSweeney *et al*., 2001). Tannins express their inhibitory effect on digestion by combining with pectin, hemicellulose, cellulose, dietary proteins and minerals (McSweeney *et al*., 2001) and express their inhibitory effect on enzymes by inactivating the enzyme through the formation of tannin-enzyme complexes (Bae *et al*., 1993). Bae *et al*. (1993) suggested that condensed tannins suppressed forage degradation in the rumen by suppressing microbial enzymes

(Bae *et al*., 1993). The effect of the condensed tannin birdsfoot trefoil on endoglucanase produced by *F. succinogenes* S85 was studied by Bae *et al*. (1993). The endoglucanase associated with the culture medium fraction was totally inhibited by condensed tannins at a concentration of >400 µg ml^{-1} (Bae *et al*., 1993). The endoglucanase fraction, associated with the cells, increased in an almost linear fashion with the initial increase in condensed tannin concentration and also displayed almost total inhibition at 400 µg. ml^{-1} of condensed tannins (Bae *et al*., 1993). Therefore, all cellulose digestion was inhibited when the condensed tannins reached a concentration of 400 µg ml^{-1} (Bae *et al*., 1993). Bae *et al*. (1993) also suggested that the presence of protein-tannin complexes on the cell surface could be an indication that the tannins not only inactivate enzymes but also interfere with their adhesion process.

Jones *et al*. (1994) investigated the effect of sainfoin leaf condensed tannins on *B. fibrisolvens* A38, *R. amylophilus* WP225, *P. ruminicola* B_14 and *S. bovis* 45S1. The condensed tannins successfully inhibited *S. bovis* 45S1 and *B. fibrisolvens* A38. Morphological changes for these two bacteria were detected by use of scanning and transmission (Jones *et al*., 1994). These changes suggested that the tannins most likely targeted the cell walls (Jones *et al*., 1994). However, condensed tannins did not have an inhibitory effect on *R. amylophilus* WP225 or *P. ruminicola* B_14 (Jones *et al*., 1994).

Cutin content. Cutin covers the aerial plant surface and epidermal cells outer wall, indirectly protecting the plant against microbial digestion by restricting the access of microbes to the fermentation sites (Orpin, 1984; Allen and Mertens, 1988). This waxy outer layer penetrates the outer polysaccharides and lamella of the cell wall (Orpin, 1984). Cutin forms a three-dimensional network of polymeric fatty acids that are interlinked by peroxide, ester and ether linkages (Orpin, 1984). Monson *et al*. (1972) showed that the presence of an intact cuticle on the surface of plant particles lead to a six to 48-hour lag time in the commencement of digestion. The cuticle effectively protected various plant species and genotypes against digestion and digestion could only take place with disruption of the cuticle (Monson *et al*., 1972). Mastication is necessary to initiate the disruption of

the cutin layer, increasing the chance of non-specific binding of cellulolytic bacteria (Miron *et al*., 2001). Additionally, all processing of feedstuffs causes disruption of the cuticle layer and will, therefore, improve digestibility (Monson *et al*., 1972).

Fat supplementation. Ruminant diets supplemented with dietary lipids are known to modify ruminal digestion (Sutton *et al*., 1982) due to their effect on the microbial population. Sutton *et al*. (1982) found that sheep fed diets supplemented with protected or free oils displayed a great depression in ruminal NDF digestibility. Linseed oil, coconut oil, protected linseed oil or protected coconut oil reduced NDF digestion by 62%, 76%, 42% and 24% respectively (Sutton *et al*., 1982). Similarly, Ueda *et al*. (2003) found that Holstein cows fed a concentrate rich diet supplemented with 3% linseed oil displayed a 22.7% reduction in ruminal NDF digestibility compared to the control. However, Doreau and Chilliard (1997) showed that Holstein cows fed a complete diet supplemented with fish oil displayed an increase in fibre digestibility.

2.4 CATABOLITE REGULATORY MECHANISMS

Catabolite repression was initially referred to as the “glucose effect” (Magasanik, 1961). The glucose effect regulates the production of certain enzymes by glucose (Magasanik, 1961). However, it was later found not to be limited to glucose. Any compound capable of efficiently assisting as a source of energy, and of intermediary metabolites, can have an inhibitory effect on the rate of production of glucose-sensitive enzymes in relation to the production of other proteins formed (Magasanik, 1961). Table 2.3 shows ruminal microorganisms that are associated with enzymes or substrates that display catabolite regulation.

Table 2.3 Identified ruminal microorganisms that are associated with enzymes or substrates that display catabolite regulation.

Microorganism	Enzyme/substrate and regulator	Reference
Selenomonas ruminantium	Maltose, lactate and cellobiose utilization by sucrose and glucose	Russell and Baldwin (1978)
Streptococcus bovis	Cellobiose utilization by sucrose Maltose utilization by glucose and sucrose	Russell and Baldwin (1978)
Butyrivibrio fibrisolvens	Glucose, cellobiose and xylose utilization by sucrose and maltose	Russell and Baldwin (1978)
Megasphaera elsdenii	Sucrose utilization by glucose and maltose	Russell and Baldwin (1978)
Ruminococcus albus	Cellulase by cellobiose	Hungate (1966)
***Neocallimastix frontalis* strain PN-1**	Xylose and fructose utilization by glucose	Mountfort and Asher (1983)
	Xylanase by xylose	Mountfort and Asher (1989)
***Neocallimastix frontalis* strain PN-2**	Endoglucanase by glucose	(Mountfort and Asher (1985)
Neocallimastix frontalis	α-Amylase by glucose	Mountfort and Asher (1988)

Catabolite regulatory mechanisms for five species of bacteria isolated from the bovine rumen were investigated by incubating each bacterium with a mixture of glucose, sucrose, xylose, maltose, cellobiose and lactate (Russell and Baldwin, 1978). Incubation of *S. bovis* with multiple substrates identified substrates which displayed catabolite regulation (Russell and Baldwin, 1978). Both sucrose and glucose inhibited maltose utilisation and sucrose suppressed cellobiose utilisation (Russell and Baldwin, 1978). Incubation of *S. ruminantium* with multiple substrates identified maltose, cellobiose and lactate as being catabolically regulated by sucrose and glucose (Russell and Baldwin, 1978). Seeing as *S. ruminantium* is a producer and utilizer of lactate, Russell and Baldwin (1978) hypothesised that the lactate dehydrogenase enzyme is subjected to catabolite regulation. Incubation

of *B. fibrisolvens* with the mixture of six substrates identified that glucose, cellobiose and xylose utilisation was inhibited by both sucrose and maltose (Russell and Baldwin, 1978). A growth lag for *B. fibrisolvens* was observed after the exhaustion of maltose and sucrose (Russell and Baldwin, 1978). *Megasphaera elsdenii* is, for the most part, a DL-lactate fermenting bacterium (Counotte, 1981). *M. elsdenii* displayed catabolite regulation for sucrose by glucose and maltose. Growth on sucrose yielded the slowest growth rate for *M. elsdenii* (Counotte, 1981). *P. ruminicola* displayed evidence of glucose production as glucose was detected in both the maintenance and incubation media that were free of added glucose (Russell and Baldwin, 1978). Additionally, *P. ruminicola* displayed sequential utilisation of the substrates, strongly suggesting the presence of a catabolite regulatory mechanism (Russell and Baldwin, 1978).

In a study by Mountfort and Asher (1983) sugar disappearance was monitored to determine the simultaneous or sequential use of various substrates. The sugars that displayed preferential utilisation was further investigated by quantitative methods (Mountfort and Asher, 1983). The utilisation of the substrates xylose and fructose by *N. frontalis* displayed catabolite regulation by glucose (Mountfort and Asher, 1983). These sugars were only utilised when glucose was almost depleted (Mountfort and Asher, 1983).

R. albus inoculated in cellulose agar containing cellobiose have been shown to partially inhibit and totally inhibit cellulase activity at 0.07% and 0.27% cellobiose respectively (Hungate, 1966). However, cellulase activity continues after the sugar concentration decreases (Hungate, 1966). Mountfort and Asher (1985) have done extensive work on the catabolite repression experienced by enzymes produced by *N. frontalis.* Glucose could play a role in the regulation of carboxymethylcellulase (endoglucanase) produced by *N. frontalis* strain PN-2 as a reverse correlation between the enzyme concentration and the substrate concentration was observed (Mountfort and Asher, 1985). Similarly, the production of xylanase by *N. frontalis* PN-1 was repressed by the presence of xylose leading to the accumulation of xylose and, to a smaller degree, by the presence of arabinose in the medium (Mountfort and Asher, 1989). The α-amylase produced by *N. frontalis*

showed production regulation of the enzyme by glucose in diauxic growth studies and glucose addition experiments (Mountfort and Asher, 1988).

Generally, the non-catabolite regulated substrates displayed a higher maximum growth rate compared to the catabolite regulated substrates (Russell and Baldwin, 1978). These researchers also indicated that catabolite regulation of substrates by species is important in the maintaining of microbial population diversity in the rumen. It is evident that bacteria and fungi make use of sequential utilisation of substrates, but to what degree? This still has a need for further investigation. Knowing and identifying the catabolite regulatory mechanisms present in the rumen can provide us with information that explains why some enzymes would have lower activity in certain conditions.

2.5 PROTEIN DIGESTION

Ruminants are the only livestock that effectively utilise nitrogen (N) by the symbiotic relationship with microorganisms in the rumen (Belanche *et al*., 2013). The protein supplied by the diet of the animal is utilised by the ruminant directly (rumen undegradable protein, RUP) and by microorganisms for the production of microbial protein (rumen degradable protein, RDP; Broderick, 2003). Rumen degradable protein is made up of true protein and nonprotein N (Bach *et al*., 2005). True protein gets digested to produce amino acids and peptides, that in turn gets deaminated to produce ammonia or gets used in the production of microbial protein (Bach *et al*., 2005). Nonprotein N is made up of N found in amino acids or small peptides, ammonia, RNA and DNA (Bach *et al*., 2005) and gets utilised for microbial growth (Bach *et al*., 2005).

2.5.1 Proteolytic enzymes

The process of ruminal protein digestion has been the subject of numerous studies as protein digestion in the rumen is discouraged as it often leads to a loss in N when RDP is in excess. However, unlike amylolytic and fibrolytic enzymes, ruminal proteolytic enzymes have not received as much attention in the literature.

Both bacteria and protozoa add to the ruminal proteolytic activity, although the bacteria produce 6 to 10 times more of the activity (Brock *et al*., 1982). Blackburn and Hobson. (1960a) fractionated rumen fluid with the use of multiple stages of sonication to separate the rumen fluid into small bacteria, large bacteria and protozoal fractions. Proteolytic activity was detected in all of these fractions. In contrast to the findings of Brock *et al*. (1982), the protozoal fractions produced the highest percentage of the total proteolytic activity followed by large bacteria and then the small bacterial fraction. However, the large bacterial fraction produced the highest activity per unit of weight. Blackburn and Hobson (1960a) suggested the reason for the contrasting results could be due to contamination of the protozoal fraction with small bacteria. Protozoa produce cysteine protease that displays optimum activity at pH 5.8, aminopeptidase activity as well as a restricted amount of deaminase activity (Forsberg *et al*., 1984). Additionally, protozoa aids in the regulation of bacterial N as well as supplying the microorganisms with soluble protein for growth (Bach *et al*., 2005).

Proteolytic activity of the anaerobic bacterium *R. amylophilus* strain H 18 (Blackburn, 1968) was measured by a modified method of the assay procedure of Anson (1938). Folin-Ciocalteu reagent was used as the colouring agent, and a spectra colourimeter was used to read the extension (Blackburn, 1968). One unit of proteolytic activity equalled the amount of enzyme required to digest 1.0 µg tyrosine in a minute (Blackburn, 1968). No protease repression was observed for a wide range of substrates (Blackburn, 1968). The bacterium secreted 20% of its protease enzymes into the culture medium and produced an enzyme that displayed trypsin-like action (Blackburn, 1968). Additionally, proteolytic activity produced optimum activities at two pH levels (6.5 and 8.0), suggesting that there were two active enzymes (Blackburn, 1968). This study led Blackburn (1968) to the conclusion that proteolysis in the rumen is not the work of one group of bacteria, but rather the joined action of various species.

The proteolytic activity produced by *P. ruminicola* R8/4 yields a mixture of various activities (Hazlewood and Edwards, 1981). Hazlewood and Edwards (1981) suggested that R8/4 displayed cysteine, serine and aspartic acid activity and that at least some of the activity was dependant on the

presence of metal ions. Russell *et al*. (1981) identified *S. bovis* as a very proteolytic bacterium, especially under high carbohydrate conditions. *S. bovis* efficiently produces microbial protein out of exogenous protein (Russell *et al*., 1981).

Prevotella is one of the main genera in the ovine rumen microbiome, representing more than 30% of the bacterial population (Lopes *et al*., 2015). Additionally, Lopes *et al*. (2015) observed a positive correlation between the quantity of *Prevotellaceae* and ruminal organic matter digestion. Griswold *et al*. (1999) performed a study on five *Prevotella* ssp. namely, *P. bryantii* $B_1$4, *P. albensis* M384, *P. brevis* GA33, *P. ruminicola* D31d and *Prevotella* sp. 2202. The N source affected the proteolytic activity and the growth rate of *Prevotella* ssp. (Griswold *et al*., 1999). *P. albensis* M384, *Prevotella* sp. 2202 and *P. bryantii* $B_1$4 all produced metalloproteases that varied in molecular weight. *P. brevis* GA33 produced a clearing zone that contained cysteine, serine and metalloproteases. *P. ruminicola* D31d produced two metalloproteases and one cysteine protease. Additionally, *P. ruminicola* M384 plays a major role in peptide degradation and in dipeptidyl peptidase activity compared to other bacterial species in the ovine rumen (Wallace, 1997).

Brock *et al*. (1982) tentatively identified metallo-, serine and cysteine-proteases as the main enzymes produced by proteolytic ruminal bacteria in cattle. Cysteine was found to be the most abundant protease in the ruminal inocula collected from steers and was unaffected by diet ($P < 0.1$; (McAllister *et al*., 1993). This was supported by Attwood and Reilly (1996) that found that cysteine was also the most abundant protease present in the bacterial fraction of rumen fluid collected from cattle on a mixed pasture diet.

2.5.2 Distribution of proteolytic enzymes

The distribution of proteolytic enzymes with regard to the liquid, digesta or microbial fraction of rumen contents has been shown to variate greatly between studies. The reason for this is, however, unclear. Most of the proteolytic activity responsible for ruminal proteolysis is connected to the microorganisms, with very little exogenic enzyme release into the liquid fraction (Blackburn and

Hobson, 1960a; b). Lee *et al*. (2002) reported that most of the protease activity of bulls fed a lucerne based diet, was associated with the liquid fraction and the microbial cells, with less associated with the particulate matter. However, Brock *et al*. (1982) determined that ±25% of the proteolytic activity of bovine rumen contents was associated with the liquid fraction. Attwood and Reilly (1996) categorised the enzymatic activity of eight strains of bacteria isolated from the rumen of cows according to their mode of hydrolysis, their cellular location and their response to protease inhibitors. The greatest part of the protease activity was associated with the cell-bound, and cell-associated fraction of the rumen fluid and no protease activity was detected in the supernatant fraction (Attwood and Reilly, 1996). An *in vitro* study was performed on bovine rumen fluid to determine the effect of enriching the inoculum with particle associated microorganisms on protein digestion (Craig *et al*., 1984). The inoculant enriched with the particle associated microorganisms displayed more than twice the rate of casein digestion compared to the normal inoculum (Craig *et al*., 1984). Although the literature has reported differences in the location of protease enzymes, adherence of microorganisms to feed particles is vital in the digestion of feed protein in the ruminant (McAllister *et al*., 1994). It, therefore, complicates proteolytic activity assays as one needs to either free the cell-bound enzymes or use whole cells (Russell *et al*., 1981). In the attempt to dislodge enzymes from cells, cell lysis could cause a loss in microbial activity or cause the release of intracellular enzymes that are normally not associated with the digestion of exogenous protein. This was observed in the study by Forsberg *et al*. (1984) where lysed protozoa displayed a substantial increase in proteolytic activity both in the absence and presence of added substrate. Additionally, some proteolytic microorganisms have the ability to form an association with one another, working in synergy to ferment protein into peptides and amino acids (Bach *et al*., 2005)

2.5.3 Factors interacting with ruminal protein digestion

The capacity of the ruminal microorganisms to produce microbial protein is often insufficient in keeping up with the rate of NH_3 production (Russell *et al*., 1992; Falconer, 1998). When RDP is in

excess, protein are digested to ammonia N and then gets absorbed (Bach *et al*., 2005). The absorbed N gets metabolised to yield urea in the liver and finally gets expelled via the urine (Bach *et al*., 2005). It is, therefore, optimum for farmers to avoid excessive protein digestion in the rumen to decrease N losses. That is why it is important to take all the aspects of the diet into account when doing ruminal proteolytic activity studies. The main factors affecting protein digestion include the interaction between protein and other nutrients, the type of protein and the dominating microbial population in the rumen (influenced by passage rate, ration composition and ruminal pH; Bach *et al*., 2005).

Type of protein. The solubility of proteins plays a major role in how susceptible proteins are to digestion by microbial proteolytic enzymes (Bach *et al*., 2005). For example, glutelins and prolamins display resistance to degradation in the rumen (Romagnolo *et al*., 1994). Globulins, on the other hand, are readily digested in the rumen due to their soluble nature (Romagnolo *et al*., 1994). Romagnolo (1994) suggested that one of the reasons for reduced degradability of proteins in the rumen could be attributed to increased hydrophobicity of some proteins. However, studies on ovalbumin degradation by ruminal microorganisms have shown that structure, and not solubility alone, effects proteolysis in the bovine rumen (Mangan, 1972). Although some albumins are soluble, they contain disulphide bonds that extend the time needed for ruminal digestion (Bach *et al*., 2005). Proteins present in the cell wall structure of plants is almost completely indigestible and can move through the gastrointestinal tracts untouched (Hatfield *et al*., 1999).

pH. Blackburn and Hobson (1960a) showed that the optimum pH for proteolysis in the rumen falls in the range of 6.0 and 7.0, with the optimum around pH 6.5.

Protease inhibitors. Protease activity of rumen fluid of bovine origin was assayed with azocasein and L-leucine-ρ-nitroanilide hydrochloride substrates separately to determine the effect of 18 protease inhibitors at three concentrations each (Brock *et al*., 1982). The Holstein cows received lucerne hay twice daily as well as a maize grain supplement (Brock *et al*., 1982). The trypsin inhibitors of plant origin identified in this study included lima bean and soybean types I-S and II-S (Brock *et al*., 1982). The protease inhibitors of microbial origin identified in this study included chymostatin,

leupeptin and antipain (Brock *et al*., 1982). Making use of plant, microbial and even chemical protease inhibitors has helped researchers identify the classes of proteolytic activity produced by an organism (Hazlewood and Edwards, 1981).

Ionophores. Monensin is a dietary ionophore that inhibits gram-positive bacteria due to the nature of their cell walls (Duffield, 2002). Ruminal NH_3 production was inhibited by the presence of monensin in the diets of fistulated sheep (Eschenlauer *et al*., 2002). Eschenlauer *et al*. (2002) did an *in vitro* study to determine the effect of monensin and substrate (at different inclusion amounts) on the production of NH_3 by mixed ruminal microorganisms. Rumen fluid was sampled from sheep maintained on a mixed hay-concentrate diet (Eschenlauer *et al*., 2002). At the inclusion level of 2 mg ml^{-1} casein was more rapidly degraded than trypticase peptone, soya peptone, soluble soybean protein or an amino acid mix based on the composition of casein (Eschenlauer *et al*., 2002). Monensin inhibited the production of NH_3 from amino acids, peptides and proteins at the inclusion level of 2 and 20 mg ml at an average of 28% and 48%, respectively. The addition of monensin had, for the most part, a bigger effect on protein breakdown than on the breakdown of peptides (Eschenlauer *et al*., 2002). All nineteen of the bacteria isolated from the ovine rumens were inhibited by the presence of monensin (Eschenlauer *et al*., 2002). All the bacterial groups identified were capable of growing on amino acids and peptides, all displaying exopeptidase activity. These proteolytic enzymes producing bacteria groups identified were most closely related to *Clostridium* sp., *Eubacterium* sp., *Atopobium minutum*, *Desulfomonas piger* and *Acidaminococcus fermentans* (Eschenlauer *et al*., 2002). Eschenlauer *et al*. (2002) determined that ammonia-hyper producing bacteria contribute 23 to 36% to the production of NH_3 because they are more sensitive to the presence of the dietary ionophore.

Condensed tannins. Condensed tannins react with the proteins within forages after mastication to form condensed tannin-protein complexes in the rumen (Min *et al*., 2003, 2005). These complexes then bypass digestion of protein in the rumen and are released in the more acidic environment of the abomasum (Min *et al*., 2005). Decreased ruminal protease activity and increased

ruminal protected protein were observed in sheep fed *Lotus corniculates* (birdsfoot trefoil), a legume containing 3.2% condensed tannins on a dry matter basis. Additionally, species-specific inhibition of proteolytic bacteria by condensed tannins was observed (Min *et al*., 2002).

Passage rate. Ørskov and McDonald (1979) demonstrated that an increase in passage rate causes a decrease in ruminal protein degradability. Feeding sheep dried grass supplemented with soybean *ad libitum* caused an increase in passage rate (k=0.060) with a final protein degradability estimate of 66% (Ørskov and McDonald, 1979). Restricted feeding had a lower passage rate (k=0.046/h) with a final protein degradability estimate of 71% (Ørskov and McDonald, 1979).

2.6 MICROBIAL INTERACTIONS

Microbe-microbe interactions between the different species of bacteria, fungi and protozoa in the rumen is essential to maintain the microbial population and its respective activities (Wolin *et al*., 1997) Interactions between microbes can be divided into parasitism, synergism and symbiosis. Microbial interactions can occur between or within microbial types (Dehority, 1998, 2003). The greatest positive effect of microbial interactions in the rumen is with regards to the synergism between bacteria and fungi and the cross feeding and synergism between bacterial species (Dehority, 1998). All other microbial interactions are regarded as parasitic in nature (Dehority, 1998). Protozoa is known to display predation towards other ciliate protozoa, bacteria and fungi (Dehority, 1998). According to Dehority (2003) even though the microbial interactions are demonstrable *in vivo*, the significance of microbial interaction *in vivo* may be very limited.

2.7 MANAGEMENT FACTORS AFFECTING ENZYMATIC ACTIVITY IN THE RUMEN

2.7.1 Dietary fibre to starch ratio

Cone *et al*. (1989) showed that the *in vitro* starch degradation percentage for cows fed a concentrate diet was constantly higher ($P < 0.05$) than cows fed a hay diet, except when incubated with popped rice and wheat feed meal, indicating that the microbial community of a cow fed a hay

diet was less adapted to the degradation of starch. The concentration of amylolytic species in the rumen is affected by the amount of starch in the feed, with a prolonged presence of amylolytic species with an increase of starch in the diet (Ortega Cerrilla and Mendoza Martínez, 2003). Similarly, the ruminal amylolytic activity of sheep have been shown to display a linear association with the amount of starch in the diet (Giesecke and Geiges, 1974). McAllister (1993) showed that the total amylolytic activity of ruminal inocula was higher ($P < 0.001$) for steers fed a grain diet than for steers fed a lucerne diet. Similarly, the percentage of cellulolytic bacteria present in the rumen decreased with an increase in the dietary proportion of concentrate (Vlaeminck *et al*., 2004).

2.7.2 Dietary protein to energy ratio

To determine the optimal energy to protein ratio for dairy cow production, Broderick (2003) examined three levels of crude protein (CP), at three levels of energy each. The different dietary CP levels were 15.1, 16.7 and 18.4% and were obtained by replacing maize with soybean (Broderick, 2003). The different dietary energy levels were represented by three different NDF levels (36, 32, and 28% on dry matter basis) and were obtained by the addition of different levels of forage (Broderick, 2003). The author found no interaction ($P \geq 0.08$) for any of the combinations between the dietary energy and CP or vice versa. The higher level of CP did positively influence dry matter intake (Broderick, 2003).

In conditions where the carbohydrate availability was low, low levels of proteolysis, growth rate and lactate accumulation were observed (Russell *et al*., 1981). In conditions where carbohydrate availability was high, a rapid increase in proteolysis and lactate accumulation was observed 3-hours into the incubation with bovine rumen fluid (Russell *et al*., 1981).

2.7.3 Processing of feedstuffs

Processing of grains for ruminal diets is mainly focussed on improving starch digestibility. Numerous properties of starch are dependent on the source of the starch and the previous treatment

thereof (French, 1973). The aim of grain processing is to improve the efficiency by which the whole digestive tract and microorganisms in the rumen utilise nutrients (Nocek and Tamminga, 1991). As previously mentioned, starch granules can be lodged in a protein matrix. This protein matrix can be damaged by processing, subsequently increasing the accessibility of starch to enzymatic digestion (Nocek and Tamminga, 1991). Gelatinization of starch can be induced by thermal, mechanical or chemical agents or by a combination of them (Rooney and Pflugfelder, 1986). Damaged starch is more susceptible to enzyme attack compared to non-damaged starch (Rooney and Pflugfelder, 1986). The degree to which starch is damaged depends on the method used, grinding conditions, the grain used and moisture content (Rooney and Pflugfelder, 1986).

The degree to which chemical treatment of feedstuffs improve or retard degradation in the rumen depends on the specific chemical and the concentration of the chemical used (Nocek and Tamminga, 1991). Processing of feedstuffs by steam flaking and popping may expose bacteria and enzymes to the inner part of the starch granule by disturbing the surface layer (Cone *et al*., 1989). When starch is steam flaked, water and heat migrate into the kernel and some swelling of starch occurs (Rooney and Pflugfelder, 1986). The moist and hot grain is then rolled causing some of the swollen granules to tear apart (Rooney and Pflugfelder, 1986). This produces a paste that binds to other materials to form a strong flake (Rooney and Pflugfelder, 1986). By steam flaking starch, the surface area and the susceptibility of the starch to enzymes greatly increase (Rooney and Pflugfelder, 1986). Pronase treatment can increase the rate of starch digestion by hydrolysing the protein matrix and increasing the surface area of starch that is in contact with glucoamylases (Rooney and Pflugfelder, 1986).

The effect of processing of rice, wheat and maize on starch degradability was studied by Cone *et al*. (1989). The starch was either popped, steam flaked or by-products of the feed industry (maize gluten feed and maize feed meal). The *in vitro* starch degradability was higher when incubated with processed feedstuffs, especially for popped rice, wheat and maize. All the fractions for tapioca displayed high digestibility. Oat starch only displayed a slight increase in digestibility with an

increasing particle size. Trei *et al*. (1970) used gas production to determine the effect of different grain processing methods on digestion *in vitro*. Gras production was significantly increased by the steam processing and flaking of barley and milo.

On the other hand, over processing of feeds that are not readily digestible otherwise, such as grasses, could cause the particles to pass through the rumen before digestion in the rumen is completed (McAllister *et al*., 1994). Therefore, it is important that mechanical processing should only be employed to the degree that the barriers inhibiting microbial attachment and penetration are damaged.

2.7.4 Particle size and feed sorting

Maulfair *et al*. (2013) showed that cows can adapt their feeding behaviour when experiencing subacute ruminal acidosis by selecting for longer forage particles and slow-fermenting starch, thereby slowing starch fermentation. However, Kmicikewycz *et al*. (2015) showed that cows preferred short particles even when experiencing subacute ruminal acidosis induced by the oversupply of grain in the diet (50:50 forage to concentrate diet).

Cone *et al*. (1989) studied the effect of different particle sizes of maize, wheat, oat, tapioca and potato starch after 6-hours incubation in rumen fluid (from a cow fed a concentrate diet) on the percentage of starch digested. For wheat, maize and potato starch the highest level of starch digestion was observed with the smallest particle size. However, all the particle fractions for tapioca displayed high digestibility. Dry matter digestibility of a total mixed ration, fed to dairy cows, tended to decrease with an increase in grass hay particle size (evaluated up to 5.84 mm; Maulfair *et al*., 2011). Maulfair (2010) found that the time spend on eating and ruminating daily increased linearly as the total mixed ration particle size increased. Therefore, sorting can be problematic as Santini *et al*. (1983), Beauchemin *et al*. (2003) and Kmicikewycz *et al*. (2015) showed that the shorter chewing time associated with smaller particle size could negatively affect saliva production. Subsequently leading

to the depression of ruminal pH, VFA's and reticuloruminal cellulolytic activity (Santini *et al*., 1983; Kmicikewycz *et al*., 2015).

As previously mentioned, the enzymatic activity of cellulolytic bacteria decreases dramatically with even the slightest decrease in ruminal pH (Russell and Wilson, 1996). Therefore, smaller particle sizes that negatively affect saliva production can lead to a depression in fibrolytic activity.

2.7.5 Feeding systems

Cone *et al*. (1989) studied the effect of mixed fermentations in comparison to the starch degradability of single feedstuffs. Nine different combinations were examined. The starch degradability of the mixed feedstuffs was compared with the value of the starch degradability of single feedstuffs. When paselli (pure starch) was one of the feedstuffs, there was a difference between the experimental starch digestibility and the calculated value when the rumen fluid donor cow was fed either hay or a concentrate containing tapioca and steam-flaked maize. The tapioca and wheat combination also displayed a significant difference ($P < 0.05$) between the experimental starch digestibility and the calculated value.

Rottman *et al*. (2015) and Ying *et al*. (2015) studied the effect of feeding dairy cows a high fibre diet during the high intake period of the day and feeding a low fibre diet during the overnight period (when intake is lower) on total tract digestibility, rumen digesta nutrient concentration, fermentation products, pH, feeding behaviour, production, hormones and plasma metabolites. Rottman *et al*. (2015) found that this type of feeding system shifted the daily pattern of fibre and starch intake and decreased the daily intake without negatively affecting body weight gain or milk yield, but failed to display other benefits. Ying *et al*. (2015) showed that feeding behaviour, and the feeding pattern and diet composition had a collective effect on ruminal fermentation products and nutrient pool size. However, feeding dairy cows a high fibre diet during the high intake period of the

day and feeding a low fibre diet during the low intake period was unsuccessful in stabilising ruminal fermentation because of the adaptiveness of feeding behaviour (Ying *et al*., 2015).

Blackburn and Hobson (1960b) examined the effect of different combinations of hay, grass, various concentrates and potato starch on proteolytic activity and the dominating bacteria in the rumen fluid. They found no relationship between diet and proteolytic activity or between the diet and the dominating bacteria present in the rumen fluid. However, Brock *et al*. (1982) suggested that the predominant proteolytic bacteria present in the rumen will vary depending on the diet of the animal and Blackburn and Hobson (1960b) determined that the proteolytic activity of sheep fed three different diets, with the difference being the source of added casein, varied with the diet. Additionally, *in vitro* studies were performed to determine the rate of amino acid disappearance upon incubation with rumen fluid collected from cows on various diets (Prins *et al*., 1979). Prins *et al*. (1979) found that there was a correlation between diet and peptide and amino acid fermentation. The effect of diet on protein digestion can mainly be prescribed to the quantity of soluble protein present in the diet McAllister (1993) showed that serine protease activity was higher ($P < 0.05$) for steers fed an alfalfa diet than for steers fed a grain diet and attributed the difference in activity to the protein matrix.

2.7.6 Feeding frequency

In a study by DeVries *et al*. (2005) cows fed multiple times increased their time spent feeding and showed a greater distribution of time spent feeding during the day. Additionally, cows fed twice a day, compared to once a day displayed a decrease in the amount of feed sorting. This could explain the results obtained by French and Kennelly (1990) showing that cows fed 12 diets a day displayed a more stable diurnal pattern in ruminal pH, compared to cows fed twice daily that displayed more drastic changes in pH.

Rottman *et al*. (2015) showed that cows fed twice a day, compared to cows fed once a day, had the ability to adapt their feeding behaviour and displayed a decrease in their daily intake as well as a change in their daily pattern of fibre and starch intake. Ying *et al*. (2015) showed that, in

combination with diet composition (low fibre vs high fibre), feeding multiple diets had an effect on the ruminal nutrient pool size and the fermentation products. The practice of feeding multiple diets a day has the potential to change the daily pattern of nutrients entering the rumen and the ruminal parameters (Ying *et al*., 2015). However, feeding behaviour is very adaptive and must, therefore, be kept in consideration (Ying *et al*., 2015).

2.8 METHODOLOGICAL DIFFERENCES AND THEIR EFFECT ON ENZYMATIC AND *IN VITRO* FERMENTATION STUDIES

2.8.1 Rumen fluid sampling time and the effect of diurnal variation and feeding management on enzymatic activity

A circadian rhythm can be defined as any internal process related to a living organism that displays an entrainable oscillation over a 24h period. Numerous physiological activities in cows have been reported by Lefcourt *et al.* (1999), Piccione *et al.* (2007) and Giannetto and Piccione (2009) to follow a circadian pattern, including glucose, urea, non-esterified fatty acids (NEFA), total lipids, total cholesterol, haemoglobin, creatinine, magnesium, phosphorus, respiratory rate and rectal temperature. Circadian patterns also continue when environmental signals are absent, as they are controlled by time-keeping mechanisms in the peripheral tissues and central nervous system (Niu *et al*., 2014). According to Takahashi *et al*. (2008) in humans the master timekeeper and pacemakers in peripheral tissues can be desynchronised by the timing of food intake, increasing the occurrence of various disorders (Bass and Takahashi, 2010). Harvatine (2012) suggested that certain management strategies employed by dairy farmers can desynchronise the master timekeeper and mammary timekeepers and possibly desynchronise absorption of nutrients and milk production, consequently decreasing milk yield. Milk production in dairy cows has also been shown to follow a circadian pattern that is sensitive to the timing of feed intake (Rottman *et al*., 2014). Under natural grazing conditions, cows display a diurnal feeding pattern where most of the feeding activity occurs during

the day, especially at sunset and sunrise (DeVries, 2013). Housed dairy cows fed a total mixed ration (*ad libitum*) once a day also displayed a diurnal feeding pattern, with most of the activity at sunrise and sunset (Shabi *et al*., 2005). However, the diurnal feeding pattern of dairy cows in a free-stall housing setup tended to be influenced by milking, feed push-up and the time of feed delivery (DeVries *et al*., 2003). The feeding time of dairy cows can retrain the daily rhythm of feeding, core body temperature, faecal NDF and iNDF concentrations, lying behavior and plasma blood urea nitrogen, insulin and glucose concentrations (Niu *et al*., 2014). Robinson *et al*. (1997) and Robinson *et al*. (2002) showed that the time of feeding protein supplements to dairy cows affected the diurnal patterns of ruminal fermentation, VFA's, and amino acids. Blackburn and Hobson (1960b) also reported that VFA's were affected by the time of feeding. Additionally, Russell *et al*. (1981) showed that the most significant increase in ammonia was observed 1 to 3-hours after feeding. In addition to protein metabolites, Cone *et al*. (1989) and Fickett and Allen (2002) showed that the extent of starch digestibility was also affected by feeding time. Cone *et al*. (1989) determined that the time of sampling rumen fluid affects the extent of starch digestion in *in vitro* experiments. The percentage of *in vitro* starch digestion increased after the last feeding and reached maximum activity after about 12-hours when the cow was fed a low-level diet (4 kg hay and 4 kg concentrate) and after more than 16-hours when fed a high-level diet (8 kg hay and 8kg concentrate). The high-level diet displayed a prolonged period of amylolytic activity as it needed a longer period for digestion. When all the available feed was digested the percentage of starch digestion decreased. In an *in vitro* study by Fickett and Allen (2002) the effect of time relative to feeding on the digestibility of corn starch was evaluated. Starch digestibility was greater after feeding versus before feeding (41.2 vs. 30.9 %/hour, $P < 0.01$). Additionally, lag time for rumen fluid collected after feeding was higher compared to rumen fluid collected before feeding (2.53 and 1.90-hours, $P < 0.01$). Fickett and Allen (2002) attributed the variation in digestibility rates on the difference in enzymatic activity of rumen fluid collected before and after feeding. Bryant and Robinson (1968) found that bacteria counts were also affected by the timing of feeding and were at their lowest and highest one and 5.5-hours after feeding

respectively. Bryant and Robinson (1968) attributed the decrease in bacteria numbers after feeding to the dilution of rumen contents by water, feed and saliva. Similarly, protozoa and fungi concentrations have also been shown to display diurnal variations (Purser and Moir, 1959; Warner, 1966) Moreover, Wales *et al*. (2004) showed that dairy cows fed twice a day displayed a recognizable diurnal variation in ruminal pH. As shown throughout this chapter, enzymatic activity is very sensitive to pH variations. There is therefore ample reason to speculate if diurnal patterns for enzymatic activity might not also exist.

2.8.2 The particle fraction vs the liquid fraction of the rumen content

As mentioned earlier, when feed is fermented using whole rumen content the fermentation rate is approximately four times higher than the rate when feed is fermented using only the liquid part of the rumen content (Hungate, 1966). Using whole rumen content is not practical, however blending of rumen fluid before straining can increase viable bacterial counts considerably (Craig *et al*., 1984). Blending of rumen fluid before straining through cheesecloth can increase the concentration of bacteria in the liquid fraction of the rumen contents, by removing some of the bacteria attached to particles (Craig *et al*., 1987b). However, Leedle *et al.* (1982) and Craig *et al*. (1984) found that blending was insufficient in removing significant amounts of microorganisms associated with the particulate fraction of the rumen content.

For enzymatic studies different enzymatic extraction methods have been employed. Lysozyme treatment and sonication has been the most widely used when pertaining to ruminal enzymatic analysis. However, the methods employed differ widely between studies with no standard method for enzyme extraction. Patra *et al*. (2006, 2010) used both lysozyme treatment and sonication to extract enzymes from the rumen fluid of buffaloes. Rey *et al*. (2012) and Lee *et al*. (2002) employed only sonication for the extraction of enzymes from the rumen fluid of dairy calves and bulls, respectively. Martin and Michalet-Doreau (1995) did not use any pre-treatment for measuring the enzymatic activity of rumen fluid collected from Jersey cows. Only Lee *et al*. (2002) and Hristov *et*

al. (1999) compared the enzymatic activity of sonicated rumen fluid to untreated rumen fluid. Sonication increases the concentration of free enzymes in the rumen fluid by causing detachment of microorganisms from particles and also by the lysis of cells (Hristov *et al*., 1999). Lysozyme treatment of rumen fluid can increase the number of enzymes available by microbial cell lysis (Hristov *et al*., 1999). However, because of cell lysis caused by both sonication and lysozyme treatment, these treatment methods result in rumen fluid not utilisable for digestibility determination but solely for the determination of enzymatic activities. In addition to causing cell death, cell lysis extracts intracellular enzymes that would usually not be involved in the digestion of extracellular substrates. Hristov (1999) found lysozyme treatment of fresh rumen fluid to be more successful in increasing the recovery of enzymes, compared to sonication. Blending is frequently used in *in vitro* digestibility studies for the extraction of adherent microorganisms (Getachew *et al*., 2000; McAllister and Sultana, 2011; Kisidayova and Jalc, 2013). However, its effect on enzymatic activity have yet to be determined and could possibly be a less intensive method for enzymatic extraction.

2.8.3 Sampling site

Craig *et al*. (1984) found that there were significantly higher numbers of bacteria in the dorsal rumen and that the number of bacteria in the ventral rumen and reticulum varied according to the diet of the cow. Martin *et al*. (1999) reported that the greatest concentration of entodiniomorph protozoa were located in the dorsal rumen of cows and was not affected by the diet or sampling time.

2.9 CONCLUSION

This review has focused mainly on identifying the major enzymes responsible for starch, fibre and protein digestion in the rumen. Bacteria are the biggest contributors to ruminal digestion. However, the role of protozoa and fungi should not be overlooked, especially the contribution of fungi to the production of extracellular enzymes. It is evident that pH is one of the biggest influencers of microbial enzymatic activity and a wide range of factors can contribute to a change in ruminal pH.

However, amylolytic activity has been shown to be more tolerable to pH variations in comparison to fibrolytic enzymes. Numerous intrinsic factors pertaining to starch, fibre and protein have been identified to effect *in vitro* digestibility, however, how these factors affect amylolytic, fibrolytic and proteolytic activity is still poorly defined and open to investigation. There is still much work to be done in better defining the dynamic between the ruminal microbes and enzymatic activity and how the enzymatic activity reacts in response to factors affecting *in vitro* digestibility - as factors such as catabolite regression have not even been considered as a source of variation in *in vitro* digestibility studies, especially also in terms of how methodological differences (time of rumen fluid collection, fraction of rumen content used, sampling site) observed between *in vitro* studies translates to enzymatic activity, as this could help define possible sources of variation when comparing the results between *in vitro* digestibility trials of the same nature.

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Table 2.1 List of ruminal enzymes, their mode of action and some of the ruminal microorganisms responsible for their production**.**

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
Amylases				
α-Amylase	Amylose Amylopectin	Matotriose, maltose and limited amounts of glucose (Ortega Cerrilla and Mendoza Martínez, 2003) Matotriose, maltose, limited amounts of glucose and mixed α-limit dextrins (Ortega Cerrilla and Mendoza Martínez, 2003)	α-1, 4 glycosidic bonds (Rooney and Pflugfelder, 1986) Endohydrolytic activity (Cotta, 1988; Mountfort and Asher, 1988)	*Ruminobacter amylophilus* H1 (Cotta, 1988) *Streptococcus bovis* JB1 (Cotta, 1988) *Butyrivibrio fibrisolvens* 49, A38 (Cotta, 1988) *Prevotella ruminicola* 23, B_14 (Cotta, 1988) *Neocallimastix frontalis* (Mountfort and Asher, 1988) *Eudiplodinium maggii* (Bełżecki *et al*., 2007) *Diploplastron affine* (Wereszka, 2012)
β-Amylase	Starch, Amylopectin or amylase	β-limit dextrin and maltose or glucose (Rooney and Pflugfelder, 1986; El-Fallal *et al*., 2012)	Terminal glucose residues (Rooney; Pflugfelder, 1986; El-Fallal *et al*., 2012	
Isoamylases	Amylopectin	Linear dextrins (Rooney and Pflugfelder, 1986)	α-1, 6 glycosidic bonds (Rooney and Pflugfelder, 1986)	
Pullulanases	Amylopectin and pullulan	Linear dextrins (Rooney and Pflugfelder, 1986)	α-1, 6 glycosidic bonds (Rooney and Pflugfelder, 1986)	*Ruminobacter amylophilus* 70 (Anderson, 1995)

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
α-Glucosidases	Glucose	Maltose and low molecular weight maltodextrins (Chesson and Forsberg, 1997)		*Lachnobacterium bovis* strain YZ 87^{T} and YZ 63 (Whitford *et al*., 2001)
Maltase	Soluble oligosaccharides and dextrin	Glucose (French, 1973)		*Entodinium caudatum* (Bailey and Howard, 1963a) *Epidinium ecaudatum* (Bailey and Howard, 1963a) *Dasytricha ruminantium* (Bailey and Howard, 1963a) *Diploplastron affine* (Wereszka, 2012)
Amyloglucosidase	Starch, Amylopectin or amylase	β-limit dextrin and maltose or glucose (Rooney and Pflugfelder, 1986; El-Fallal *et al*., 2012)	Terminal glucose residues (Rooney; Pflugfelder, 1986; El-Fallal *et al*., 2012	*Neocallimastix frontalis* (Pearce and Bauchop, 1985)
Amylopectinase	Amylopectin	Amylose (Chudzik, 2007)	α-1,6 linkages (Chudzik, 2007; Hoffman, 2009)	*Ruminobacter amylophilus* 70 (Anderson, 1995)

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
Cellulases				
Endoglucanase (Unspecified)	Cellulose	Cellodextrins (Orpin, 1984) Cello-oligosaccharides of various lengths (Lynd *et al*., 2002)	Glycosidic linkages (Orpin, 1984)	*Bacteroides succinogenes* (Groleau and Forsberg, 1981) *Fibrobacter succinogenes* S85 (Bae *et al*., 1993) *Orpinomyces sp.* strain *PC-2* (H. Chen *et al*., 1998) *Ruminococcus albus* (Ohara, 2000) *Orpinomyces joyonii* (Qiu *et al*., 2000)
β-Glucanase	β-Glucan, cellulo-oligosaccharides, CMC and soluble xylan (Zhou *et al*., 1994)			*Neocallimastix patriciarum* (Xue *et al*., 1992; Zhou *et al*., 1994)
Endoglucanase	Cellulose			*Neocallimastix frontalis* (Mountfort and Asher, 1985) *Bacteroides succinogenes* (Huang and Forsberg, 1987)
Cellobiohydrolase (Exoglucanase)	Cello-oligosaccharides	Cellobiose (Lynd *et al*., 2002)	Non-reducing ends of the polysaccharide chain (Chesson, 1981)	*Neocallimastix patriciarum* (Xue *et al*., 1992) *Orpinomyces* sp. Y102 (Y. Chen *et al*., 2014)
Cellodextrinase (Exoglucanase)	Cellodextrins	Cellobiose or glucose (Berger *et al*., 1990)		*Bacteroides succinogenes* (Huang and Forsberg, 1987) *Butyrivibrio fibrisolvens* H17c (Berger *et al*., 1990)

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
β-Glucosidase (Cellobiase)	Cellobiose Cellodextrins	Glucose (Groleau and Forsberg, 1981)		*Neocallimastix frontalis* (Mountfort and Asher, 1985; Pearce and Bauchop, 1985) *Bacteroides succinogenes* (Groleau and Forsberg, 1981; Huang and Forsberg, 1987) *Lachnobacterium bovis* strain YZ 87^T and YZ 63 (Whitford *et al*., 2001)
Hemicellulases				
α-Galactosidase	Melibiose and oligosaccharides containing α-(1→6)-linked galactose *(*Bailey, 1963; Bailey and Howard, 1963b*)*		α-(1→6)-linked galactosides (Mital *et al*., 1973)	*Streptococcus bovis* (Bailey, 1963) *Epidinium ecaudatum* (Bailey and Howard, 1963b) *Lachnobacterium bovis* strain YZ 87^T and YZ 63 (Whitford *et al*., 2001) *Fibrobacter succinogenes* S85 (Jun *et al*., 2007)
Mannanase	Glucomannans Galactomannans Galactoglucomannans	Short chain β-1,4-manno-oligosaccharides (van Zyl *et al*., 2010)	β-D-1,4-mannopyranosyl linkages *(*Stålbrand *et al*., 1993*)*	*Fibrobacter succinogenes* S85 (Jun *et al*., 2007; Suen, 2011)
Licheninase	Lichenin β-glucan	Lichenin: pentasaccharides and trisaccharides β-glucan: tetrasaccharide and trisaccharide (Erfle *et al*., 1988; H. Chen *et al*., 1997)		*Bacteroides succinogenes* (Erfle *et al*., 1988) *Orpinomyces* sp. strain PC-2 (H. Chen *et al*., 1997) *Fibrobacter succinogenes* S85 (Jun *et al*., 2007; Suen, 2011)

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
Xylanases (Undefined)	Xylan	Xylobiose and xylo-oligosaccharides (Mountfort and Asher, 1989)	Endo-hydrolytic activity (Mountfort and Asher, 1989)	*Butyrivibrio fibrisolvens* (Hespell *et al*., 1987) *Neocallimastix frontalis* PN-1 (Mountfort and Asher, 1989) *Neocallimastix patriciarum* (Xue *et al*., 1992) *Polyplastron multivesiculatum* (Devillard *et al*., 1999; Devillard *et al*., 2003) *Ruminococcus albus* (Ohara, 2000) *Fibrobacter succinogenes* S85 (Jun *et al*., 2007; Suen, 2011)
Endoxylanase	Xylan	Xylooligosaccharides (Y. E. Lee *et al*., 1993)	Xylan backbone (Lagaert *et al*., 2014)	*Ruminococcus albus* 8 (Moon *et al*., 2011)
β-Xylosidase	Xylobiose Xylooligosaccharides	Xylose (S. F. Lee and Forsberg, 1987)		*Neocallimastix frontalis* (Garcia-Campayo and Wood, 1993) *Ruminococcus albus* 8 (Moon *et al*., 2011)
α-L-Arabinofuranosidases	Arabinoxylan		Arabinose side chains (Lagaert *et al*., 2014)	*Ruminococcus albus* 8 (Greve *et al*., 1984; Moon *et al*., 2011) *Butyrivibrio fibrisolvens* GS113 (Hespell and O'Bryan, 1992)

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
α-Glucuronides	Xylan		Residue branches linked via α-1,2-glycosidic bonds to the main xylan chain (Biely *et al*., 2000)	*Piromonas communis* (T. Wood and Wilson, 1995) *Ruminococcus albus* 8 (Moon *et al*., 2011)
Acetylxylan Esterase	Arabinoxylan		Ester bonds *(Blum et al., 1999)*	*Fibrobacter succinogenes* S85 *(McDermid et al., 1990)* *Neocallimastix patriciarum* (Dalrymple, 1997) *Orpinomyces* sp. Strain PC-2 *(Blum et al., 1999)*
Pectinases				
Pectinesterases	Pectin	Demethylated pectin and methanol (Wright, 1960)	Methyl ester groups (Abou Akkada and Howard, 1961; Aspinall, 1980)	Oligotrich and holotrich protozoa, including *Isotricha intestinalis* containing *Isotricha prostoma* and *Dasytricha ruminantium* (Wright, 1960; Abou Akkada and Howard, 1961) *Prevotella ruminicola* *Butyrivibrio fibrisolvens* *Lachnospira multiparus* (Dehority, 1969)

Enzyme	Substrate	End product	Site of action	Some of the known microbial producers
Polygalacturonases	Pectin Polygalacturonic acid	Low oligouronides and galacturonic acid (Wright, 1960; Abou Akkada and Howard, 1961)	C-1-O bonds (Aspinall, 1980) Glycosidic linkages (Abou Akkada and Howard, 1961)	Oligotrich and holotrich protozoa, including *Isotricha intestinalis* containing *Isotricha prostoma* and *Dasytricha ruminantium* (Wright, 1960; Abou Akkada and Howard, 1961) *Neocallimastix sp.* strain H15 (Kopečný and Hodrová, 1995)

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CHAPTER 3

RUMEN FLUID HANDLING AFFECTS MEASUREMENTS OF ITS ENZYMATIC ACTIVITY AND *IN VITRO* DIGESTIBILITY

3.1 ABSTRACT

Traditionally, rumen fluid is used excluding the particulate matter when *in vitro* studies are done. However, a higher concentration of rumen microbes is associated with the whole particulate fraction compared to the liquid fraction of the rumen content (Forsberg and Lam, 1977; Craig *et al*., 1987a). Microorganisms attached to feed particles tend to be retained in the rumen longer than the fluid, allowing for an increased reaction time between the substrates and enzymes (Wang and McAllister, 2002). Therefore, microorganisms associated with the particulate fraction play a bigger role in the digestion of feed in the rumen (McAllister *et al*., 1994). The aim of this study was to determine if blending or sonication could increase endoglucanase, xylanase, amylase, lichenase and proteinase activity of rumen fluid and to determine the effect of blending rumen fluid on *in vitro* digestibility of starch, neutral detergent fibre and protein. Both blending and sonication had the ability to consistently release more amylase, lichenase, endoglucanase and xylanase enzymes. However, blending was not able to release more protease enzymes and sonication cannot be replaced by blending for the extraction of cell-associated protease enzymes. For rumen *in vitro* digestibility, blending was not successful in liberating amylolytic microorganisms or enzymes associated with the particulate fraction and might have negatively affected the microbial population responsible for fibre and protein digestion. Therefore, as opposed to what is sometimes suggested, blending rumen fluid before an *in vitro* trial does not necessarily result in higher digestibility values and reduced lag (i.e. higher microbial activity) and could result in opposite results.

3.2 INTRODUCTION

In vitro systems using rumen fluid are the oldest, and still the most common laboratory methods in use for estimating digestibility in ruminant feeds. The methods used are by no means established as fixed procedures, with the methodological sequences and treatments being highly dependent on the purpose for which the assay is intended. Results obtained from *in vitro* digestibility assays, with various modifications, are routinely used in rationing models to characterise feeds and to formulate diets in order to satisfy animal requirements. In dairy cows, for example, rate and extent of digestion of neutral detergent fibre (NDF) and starch have been used in the last decade, and it has improved the way we optimiza rations. More recently, *in vitro* assays have also become more popular for the determination of protein digestibility (Ross *et al*., 2013; Evans *et al*., 2015), both ruminally and intestinally, especially because of the increased cost of protein sources and to decrease the environmental impact of nitrogen. Using rumen fluid in fermentational *in vitro* systems has the potential to estimate the ruminal *in vivo* rates of digestibility and fermentation patterns (Craig *et al*., 1984), assuming the only limiting factor during fermentation is the substrate itself. Therefore, the estimation of polysaccharide and proteolytic activity plays an important role in quantifying the fermentational capacity of the rumen. Relatively little concern has been expressed over the effect of the handling of the liquor once harvested. The microflora in the rumen, and respective enzymes in the rumen fluid, are the essential reagents of an *in vitro* assay. Therefore, handling effects of rumen fluid should not be underestimated, especially because of the assumption that enzymatic activity is not limiting within the artificial micro-environment created.

As mentioned, rumen fluid is traditionally used excluding the particulate matter. However, a higher concentration of rumen bacteria is associated with the whole particulate fraction compared to the liquid fraction of the rumen content (Hungate, 1966). Similarly, Craig *et al*. (1987a) found that 70 to 80% of the microbial mass was associated with the particulate fraction. Forsberg and Lam (1977) found that approximately 75% of ATP of microbial origin was associated with the particulate

fraction of the rumen contents in a cow fed a hay based diet. This can possibly be attributed to the fact that bacteria are usually surrounded by extracellular structures, mostly fibrous polysaccharide glycocalyx (Cheng *et al*., 1977, 1981). These extracellular slime layers can assist in the adhesion of bacteria to surfaces and possibly also enclose enzymes produced by bacteria in these structures (Cheng *et al*., 1977, 1981). In *Ruminococcus* species, protuberance-like structures have been identified on the cell surface, similar to the structures of *Clostridium thermocellum* associated with cellulosomes. It seems like fibrolytic enzymes of *Ruminococcus* species are organised into these cellulosomes structures as well (Weimer *et al*., 2009). These cellulosomes aid in the adhesion of bacteria to insoluble substrates (Lamed *et al*., 1987). Microorganisms attached to feed particles tend to be retained in the rumen longer than the fluid, allowing for an increased reaction time between the substrates and enzymes (Wang and McAllister, 2002). Therefore, microorganisms associated with the particulate fraction play a bigger role in the digestion of feed in the rumen than the free-floating organisms (McAllister *et al*., 1994).

Often *in vitro* digestibility studies are compared by adjusting forage test values based on the digestion coefficient of standards included in the studies (Milchunas and Baker, 1982). However, Milchunas and Baker (1982) found that the relative digestibilities would be biased by this procedure, because the difference in the proportions of available digestible fibre of two forages, of equal digestibility, would cause a difference in the fermentative capabilities of the different inoculums. Therefore, determining the optimal way to handle rumen fluid for *in vitro* digestibility studies to obtain the highest possible activity would be beneficial in creating a standardised procedure for *in vitro* digestibility studies of the same nature.

When feed is fermented using whole rumen content, the fermentation rate is approximately four times higher than the rate when feed is fermented using only the liquid part of the rumen content (Hungate, 1966). It is difficult to obtain a representative sample for analysis of enzymatic activity, due to the difference in activity between the liquid and solid fractions of rumen fluid (Hristov *et al*., 1999). Using whole rumen content is not practical but blending of rumen fluid before straining can

increase viable bacterial counts considerably (Craig *et al*., 1984). The rumen inocula for *in vitro* digestibility studies are usually prepared by straining through cheesecloth layers. However, blending of rumen fluid before straining through cheesecloth can increase the concentration of bacteria in the liquid fraction of the rumen contents by liberating some of the bacteria attached to the particles (Craig *et al*., 1987b). However, Craig (1984) found that blending rumen liquor without the addition of particle-associated organisms was insufficient in improving NDF and protein digestibility. When adding particle-associated organisms to the inoculum the particles could be a source of increased variation, further complicating comparing results between *in vitro* studies.

Analysing the enzymatic activity of rumen fluid in correlation with *in vitro* digestibility studies can possibly provide a different perspective to the changes observed in ruminal starch, NDF and protein digestibility. There is limited research done on the enzymatic activity of rumen fluid, and therefore there is no standard method for analysing it. Sonication and lysozyme treatment are other popular methods used to free enzymes associated with the particulate fraction of rumen fluid. Patra *et al*. (2006, 2010) used both lysozyme treatment and sonication to extract enzymes from the rumen fluid of buffaloes. Rey *et al*. (2012) and Lee *et al*. (2002) employed only sonication for the extraction of enzymes from the rumen fluid of dairy calves and bulls, respectively. Martin and Michalet-Doreau (1995) did not use any pre-treatment for measuring the enzymatic activity of rumen fluid collected from Jersey cows. Only Lee *et al*. (2002) and Hristov *et al*. (1999) compared the enzymatic activity of sonicated rumen fluid to untreated rumen fluid. Sonication increases the concentration of free enzymes in the rumen fluid by causing detachment of microorganisms from particles and also by the lysis of cells (Hristov *et al*., 1999). Lysozyme treatment of rumen fluid can increase the number of enzymes available by microbial cell lysis (Hristov *et al*., 1999). However, because of cell lysis caused by both sonication and lysozyme treatment, these treatment methods result in rumen fluid not utilisable for digestibility determination but solely for the determination of enzymatic activities. In addition to causing cell death, cell lysis extracts intracellular enzymes that would usually not be involved in the digestion of extracellular substrates. Hristov (1999) found lysozyme treatment of fresh

rumen fluid to be more successful in increasing the recovery of enzymes, compared to sonication. However, lysozyme treatment is a laborious process, and the carbon tetrachloride needed for the treatment is not always easily obtainable. Therefore, blending and sonication are more practical enzyme extraction methods.

The aim of this study was, therefore, to determine if blending or sonication could increase endoglucanase, xylanase, amylase, lichenase and proteinase activity of rumen fluid, to determine the effect of blending rumen fluid on *in vitro* digestibility of starch, NDF and protein and to determine the effect of using rumen fluid collected from individual cows.

3.3 MATERIALS AND METHODS

Rumen fluid was sampled by hand from two rumen fistulated, lactating Holstein cows before the morning feeding at the Welgevallen experimental farm of Stellenbosch University. The fluid was obtained from the ventral area of the rumen of the two cows (Cow 1: 601 DIM, 663 kg, 5.3 years old, 29.21 L average daily milk production and Cow 2: 203 DIM, 644 kg, 5.1 years old, 25.06 L average daily milk production).The ventral area of the rumen was used as sampling site because the objective of the study was to determine the activity of the fluid in addition to the fact that the parameters tested required a large amount of fluid to be analysed. Weimer *et al*. (2010) and Welkie *et al*. (2010) has shown that the bacterial community composition is distinctive to individual cows. Therefore, the fluid of each cow was treated separately to investigate the effect of using the liquor from individual cows. The cows received a total mixed ration (TMR) with maize as the main source of starch and lucerne and wheat straw as the main sources of NDF. Preliminary assays (analysed under the same conditions) displayed too much variation and subsequently feed was removed approximately 8-hours before rumen fluid was collected to reduce variability of rumen fluid caused by natural feeding behaviour before the sampling. All the procedures used in this study were approved by the Research Ethics Committee for Animal Care and Use of Stellenbosch University (SU-ACUD14-00052).

3.3.1 Determination of enzymatic activities

Rumen fluid samples for the enzymatic trial were placed on ice at sampling to slow down microbial activity. The rumen fluid was then filtered through a 2 mm sieve into a pre-cooled Erlenmeyer flask. Thereafter the rumen fluid for each cow was split into three subsamples at the laboratory. The control sample was filtered gravitationally through four layers of cheesecloth, glass wool and two layers of 200 µm porosity mesh. For the blended sample, 300 ml of rumen fluid was blended in a pre-cooled industrial blender for 1-minute on high and then filtered as per control. For the sonication sample, 13 ml of rumen fluid was sonicated on ice using a Misonix Sonicator S-4000 (Qsonica, USA) at an amplitude of 20 for six cycles of 30 s followed by 30 s rest and filtered as per control. All samples were centrifuged at 16 000 × g for 15-minutes, and only the supernatant was used for enzymatic analysis. Enzymatic assays followed immediately and samples were maintained on ice.

Enzymatic activities of amylase, endoglucanase, lichenase and xylanase were assessed colourimetrically by measuring the amount of reducing sugars released using dinitrosalicylic acid (DNS; Miller, 1959). Amylolytic activity was determined using 0.2% (w/v) cooked maize starch (S4126, Sigma-Aldrich, USA) resuspended in 0.05 M citrate buffer (pH 6.0). Endoglucanase activity was determined using 0.1% (w/v) lichenan (L6133, Sigma-Aldrich, USA) and 1% (w/v) carboxymethyl cellulose (CMC; C5678, Sigma-Aldrich, USA). Enzymatic activity measured using CMC and lichenan as substrates is referred to as endoglucanase and lichenase activity, respectively. Endoxylanase activity was determined using 1% (w/v) Beechwood xylan (X4252, Sigma-Aldrich, USA) resuspended in the same buffer. Enzymatic activities were determined by measuring the amount of reducing sugars released from substrates during 15-minutes at 39°C with 450 µl of substrate and 50 µl of enzyme sample. The mixture was boiled with 750 µl DNS for 15-minutes to stop the reaction. Spectrophotometry was used to quantify the reducing sugars at 540 nm. One unit was determined as 1 µmol reducing sugar liberated per minute using glucose or xylose as standard.

Protease activity was assessed colourimetrically by measuring the amount of azo dye released from 2% azocasein (A-2765, Sigma-Aldrich, USA) in a 0.1 M sodium phosphate buffer (pH 6.8) as per the procedure by van de Vyver *et al*. (2004). Protease activity was defined as optical density per minute and was determined by subtracting the optical density of the blank from the optical density of the test sample and dividing that value by the total incubation time (in minutes). All activities were measured in duplicate for each of three runs in total. Runs were in three successive days.

3.3.2 In vitro fermentations

Control and blended rumen fluids were used for *in vitro* starch digestibility (ivSd), neutral detergent fibre digestibility (NDFd) and nitrogen digestibility (Nd) using maize, oat hay and soya oilcake, respectively. All the substrate samples were milled through a 1 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Before milling, oat hay was first oven dried at 60°C for 48-hours. All the substrates were analysed for dry matter (DM; AOAC, 2002, Method 934.01). Residual starch was analysed as described by Hall (2000). Residual NDF was analysed as described by Raffrenato and Van Amburgh (2011), with the addition of amylase and sodium sulphite anhydrous. Nitrogen digestibility was quantified as per the rumen digestibility procedure by Ross *et al*. (2013) and the crude protein (CP), used for the determination of Nd, was determined using a Nitrogen Gas Analyzer FP528 (LECO Africa Pty Ltd, Kempton Park).

Rumen fluid samples for the *in vitro* digestibility trial were placed in a pre-warmed insulated flask at collection. The rumen fluid was then filtered through a 2 mm sieve into a pre-warmed Erlenmeyer flask. For the control, rumen fluid was filtered into another pre-warmed Erlenmeyer flask through four layers of cheesecloth, glass wool and two layers of 200 µm porosity mesh. For the blended samples, 300 ml of rumen fluid was blended in a pre-warmed industrial blender for 1-minute on high, followed by filtration as per control. Carbon dioxide was flushed into the flasks to displace any air. Substrate samples were weighed into 125 ml Erlenmeyer flasks, and 40 ml *in vitro* buffer medium (adapted from Goering and Van Soest, 1970) was added to each sample. The flasks were

then placed in a water bath (39°C) and flushed with CO_2. Flask preparation was done before rumen fluid sampling as to allow the buffer medium to reach temperature before adding the rumen fluid. Rumen fluid (10 ml) was injected into each flask using a pre-warmed automatic syringe. Inoculations were always done within 15-minutes after collection. The control and blended samples for ivSd were incubated for 7-hours. The *in vitro* NDFd samples were incubated for 6, 12 and 24-hours. The Nd samples were incubated for 16-hours. All samples were incubated in duplicate, and all fermentations were completed across five runs.

3.3.3 Statistical analysis

In vitro NDF, starch and N digestibility values, amylase, endoglucanase, xylanase, lichenase and protease activities were analysed as response variables by the GLIMMIX procedure of SAS (version 9.3; SAS Institute, Inc., Cary, NC) using a factorial arrangement of treatment method, cow, time point (for NDFd only) and their interactions. The three-way interaction (cow x method x time), only applicable for *in vitro* NDFd, was removed from the model as it was non-significant, not biologically relevant and its presence did not result in an improvement of the Bayesian Information Criterion (Schwarz, 1978; Kass and Raftery, 1995). Differences between the treated rumen fluid and the control were declared significant at $P \leq 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P \leq 0.10$ were considered tendencies. The results of treatments are reported as least squares means.

3.4 RESULTS

3.4.1 Amylolytic activity

For amylolytic activity, the effect of treatment of rumen fluid was significant ($P < 0.0001$). The least squares means of the effect of treatment on amylolytic activity are shown in Figure 3.1. As expected, amylolytic activity increased with the processing of rumen fluid ($P < 0.0001$). The amylolytic activity of blended ($P < 0.0001$) and sonicated ($P < 0.0001$) rumen fluid was significantly

higher than the activity of the control. However, amylolytic activity for blending did not differ significantly from that of sonication ($P = 0.5803$). The effect of cow was also significant ($P = 0.0003$). The least squares means for the effect of cow on amylolytic activity are shown in Figure 3.2. The amylolytic activity of cow 1 was significantly higher than that of cow 2 ($P = 0.0003$).

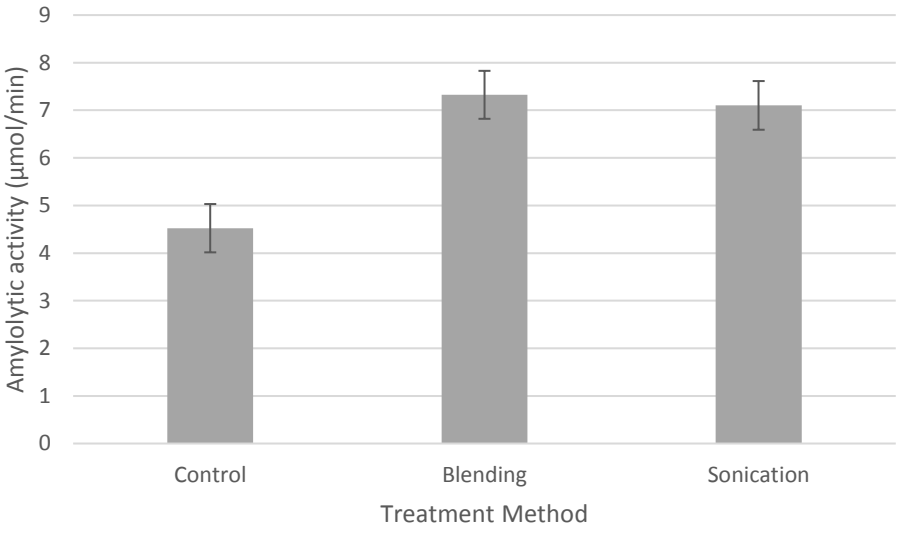

Figure 3.1 Least squares means of the effect of treatment on amylolytic activity.

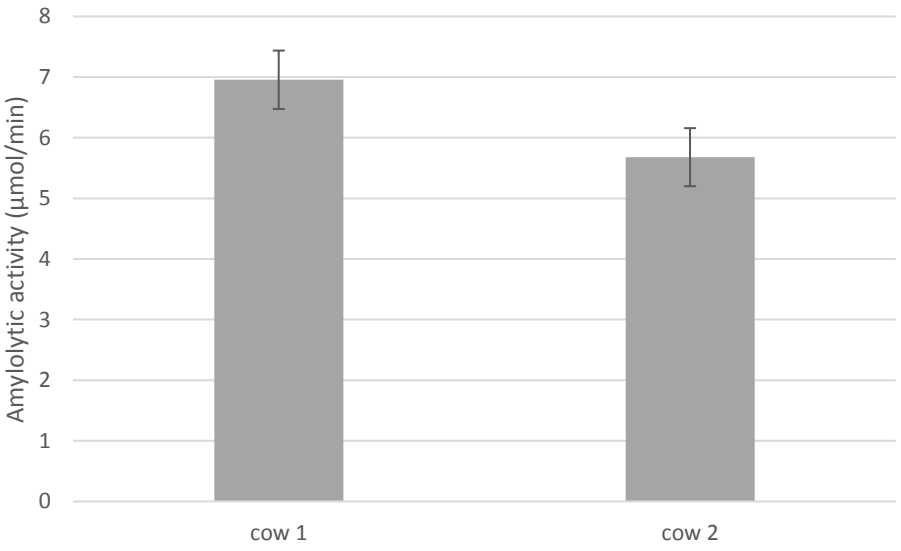

Figure 3.2 Least squares means of the effect of cow on amylolytic activity.

For amylolytic activity, the cow by treatment interaction was also significant ($P = 0.0258$). The least squares means of the effect of cow by treatment interaction on amylolytic activity are shown

in Figure 3.3. The amylolytic activity of the control sample differed significantly between the cows ($P < 0.0001$), with cow 1 having almost double the amylolytic activity when compared to cow 2. However, the amylolytic activity of blending ($P = 0.2335$) and sonication ($P = 0.3246$) did not differ between the cows.

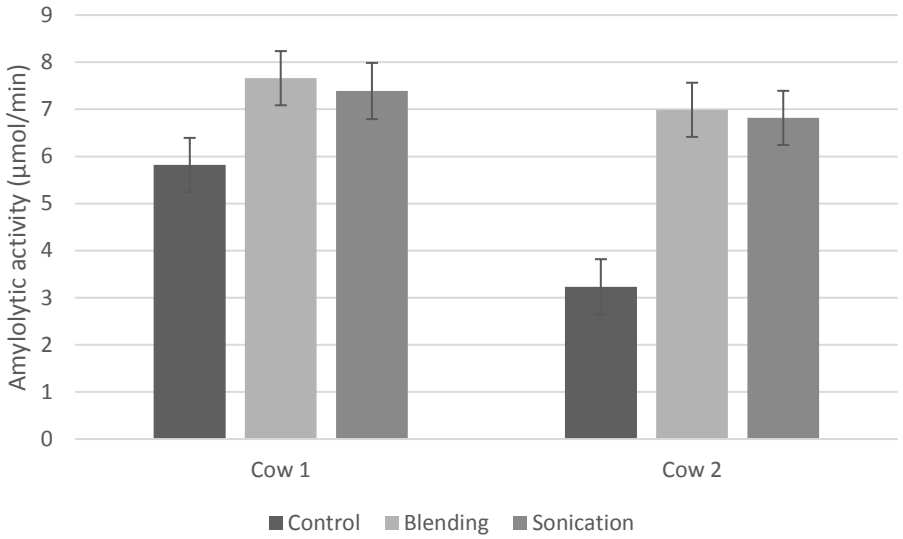

Figure 3.3 Least squares means for the effect of cow by treatment interaction on amylolytic activity.

3.4.2 Lichenase activity

The effect of treatment of rumen fluid on lichenase activity was significant ($P < 0.0001$). The least squares means for the effect of treatment method on lichenase activity are shown in Figure 3.4. The lichenase activity of the control was significantly lower than both blending ($P < 0.0001$) and sonication ($P < 0.0001$). However, there was no significant difference between blending and sonication ($P = 0.4405$). The effect of cow on lichenase activity was not significant ($P = 0.7233$). There was, however, a significant interaction between cow and treatment ($P < 0.0001$). The least squares means for the effect of cow by treatment interaction on lichenase activity can be seen in Figure 3.5.

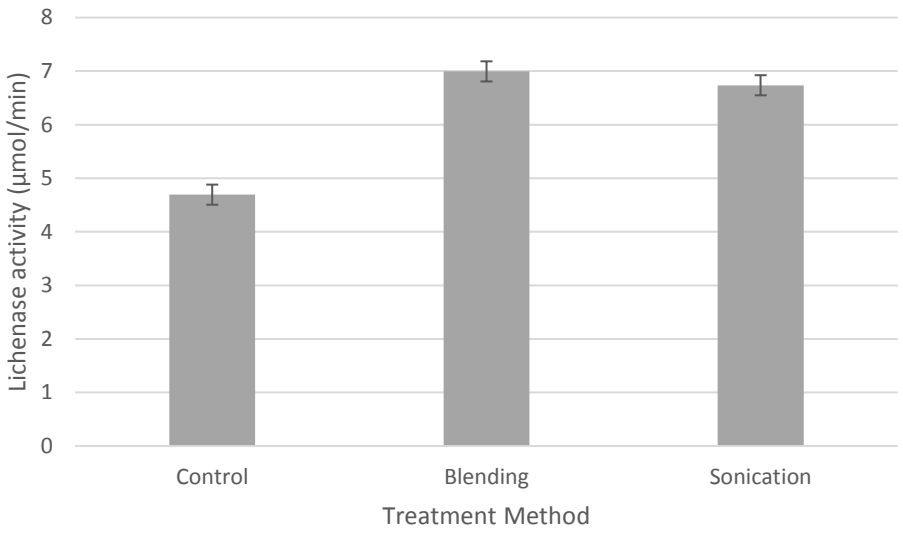

Figure 3.4 Least squares means for the effect of treatment on lichenase activity.

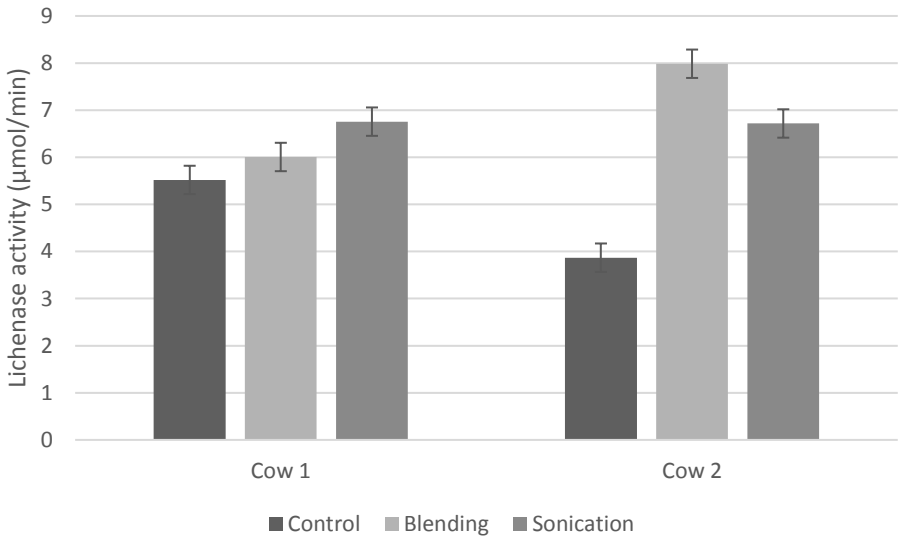

Figure 3.5 Least squares means for the effect of cow by treatment interaction on lichenase activity.

For the control, the lichenase activity of cow 1 was significantly higher than the activity of cow 2 ($P = 0.0010$). Similarly, for blending, the lichenase activity of cow 2 was significantly higher than the activity of cow 1 ($P < 0.0001$). However, there was no significant difference between the two cows in sonication ($P = 0.9339$).

3.4.3 Endoglucanase activity

The effect of treatment of rumen fluid on endoglucanase activity was significant ($P = 0.0001$). The least squares means for the effect of treatment method on endoglucanase activity are shown in Figure 3.6. The endoglucanase activity of the control was significantly lower than the activity for blending ($P < 0.0001$) and sonication ($P = 0.0003$). However, there was no significant difference between the endoglucanase activity of blending and sonication ($P = 0.6987$). The endoglucanase activity for cow 1 was significantly higher than the activity for cow 2 ($P < 0.0001$). The least squares means for the effect of cow on endoglucanase activity are shown in Figure 3.7.

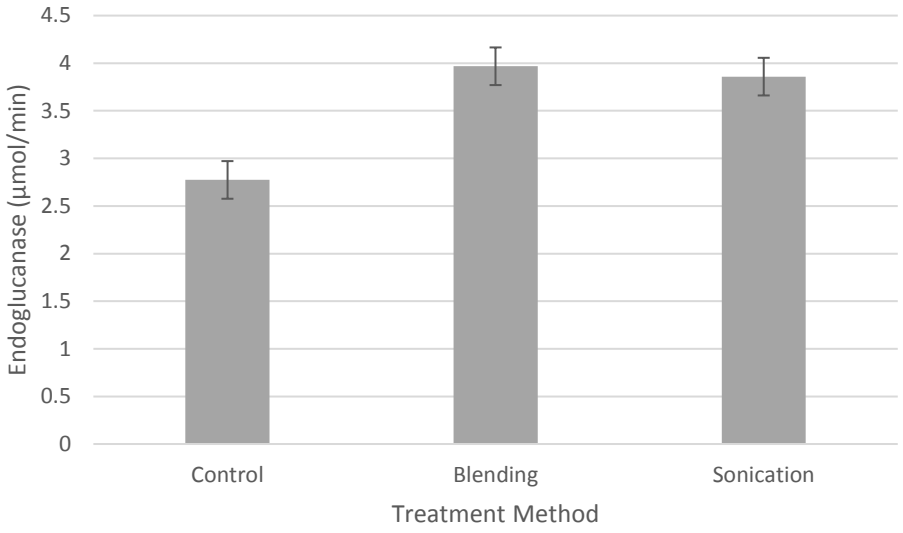

Figure 3.6 Least squares means of the effect of treatment method on endoglucanase activity.

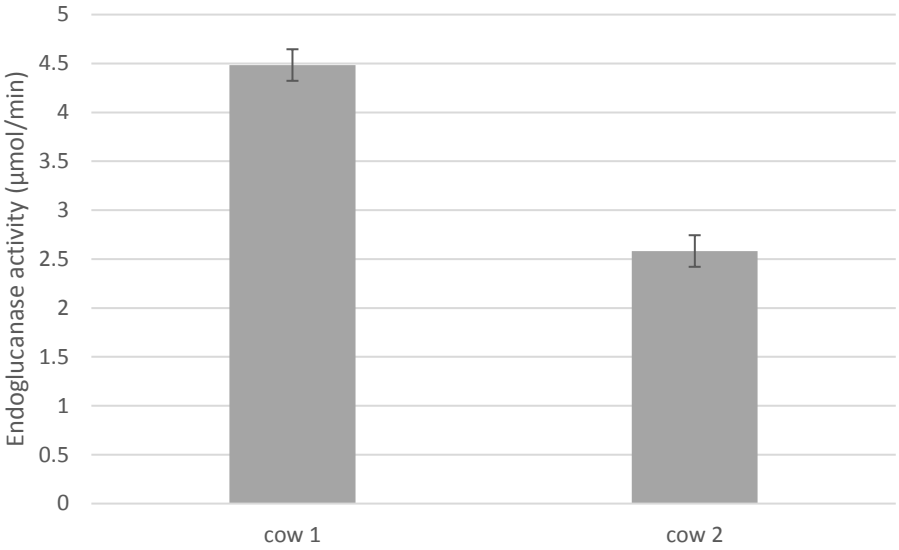

Figure 3.7 Least squares means for the effect of cow on endoglucanase activity.

There was a significant cow by treatment interaction for endoglucanase activity ($P = 0.0006$). The least squares means for the effect of cow by treatment interaction on endoglucanase activity are shown in Figure 3.8. There was a significant difference between the two cows for control ($P < 0.0001$) and sonication ($P < 0.0001$), with cow 1 being higher for both control and sonication. However, there was no significant difference between the two cows for blending ($P = 0.1407$).

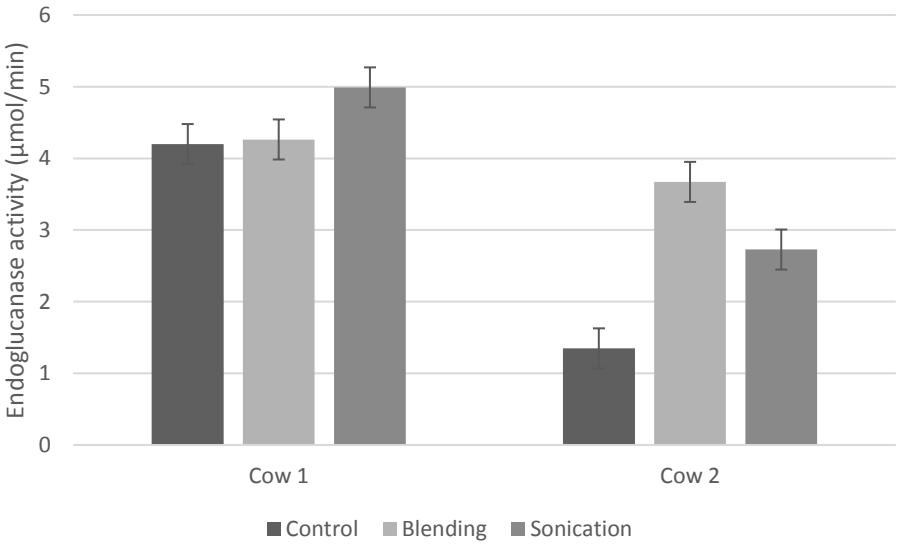

Figure 3.8 Least squares means for cow by treatment interaction for endoglucanase activity.

3.4.4 Xylanase activity

The effect of treatment method of rumen fluid on xylanase activity was significant ($P < 0.0001$). The least squares means for the effect of treatment on xylanase activity are shown in Figure 3.9. The xylanase activity for the control was significantly lower than the activity for blending ($P = 0.0006$) and sonication ($P < 0.0001$). However, there was no significant difference between the xylanase activity of blending and sonication ($P = 0.1896$).

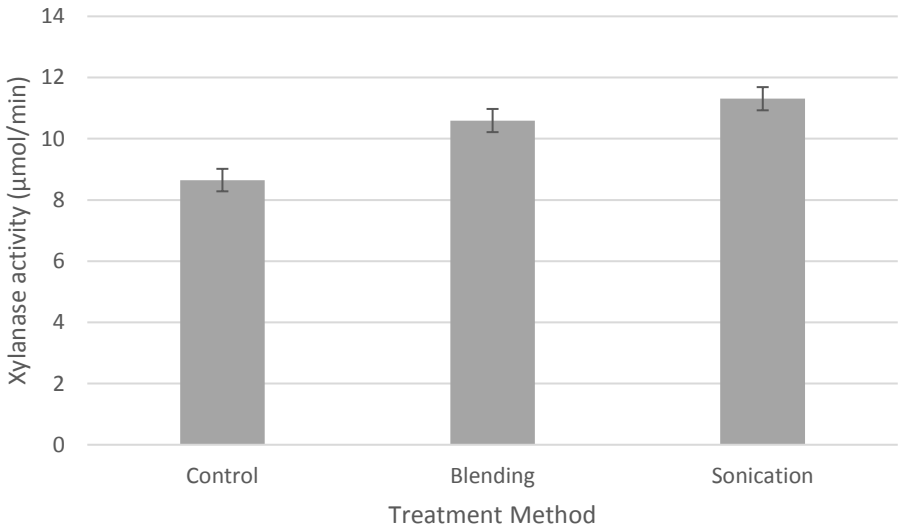

Figure 3.9 Least squares means for the effect of treatment method on xylanase activity.

Cow 1 had a significantly higher xylanase activity than cow 2 ($P < 0.0001$). The least squares means for the effect of cow on xylanase activity are shown in Figure 3.10. There was a significant effect of cow by treatment interaction on xylanase activity ($P = 0.0006$). The least squares means for the effect of cow by treatment interaction are shown in Figure 3.11. The xylanase activity for cow 1 was significantly higher than cow 2 for control ($P < 0.0001$), blending ($P < 0.0001$) and sonication ($P < 0.0001$).

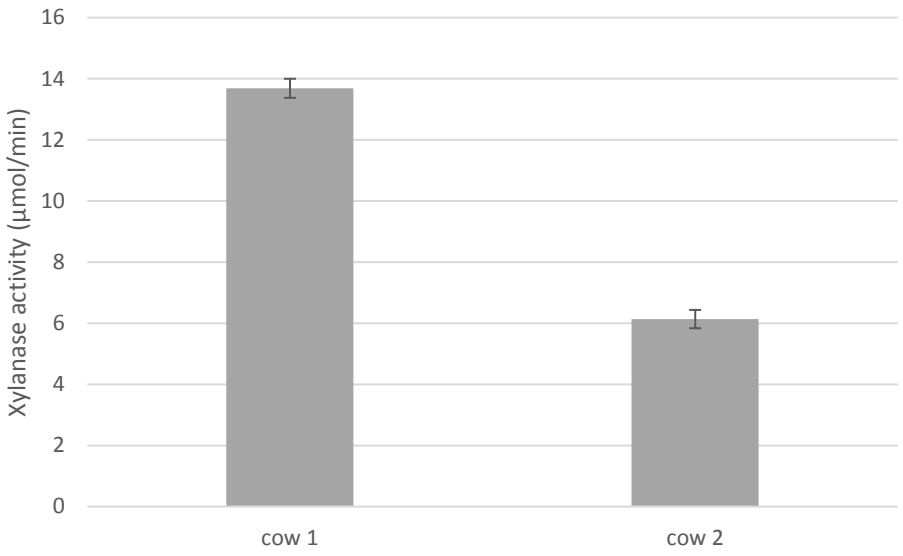

Figure 3.10 Least squares means for the effect of cow on xylanase activity.

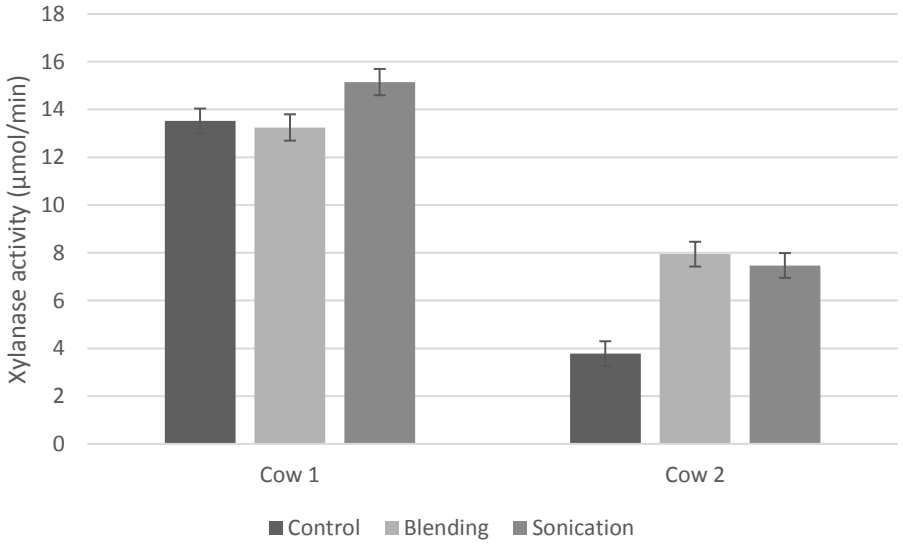

Figure 3.11 Least squares means for the effect of cow by treatment interaction on xylanase activity.

3.4.5 Protease activity

There was a significant effect of treatment method on protease activity ($P = 0.0026$). The least squares means for the effect of treatment method on protease activity can be seen in Figure 3.12. There was no significant difference between the control and blended rumen fluid ($P = 0.1515$) However, the protease activity of the control was significantly lower than the activity of the sonicated

rumen fluid (P = 0.0007). The activity of protease for blending and sonication also differed significantly (P = 0.0322).

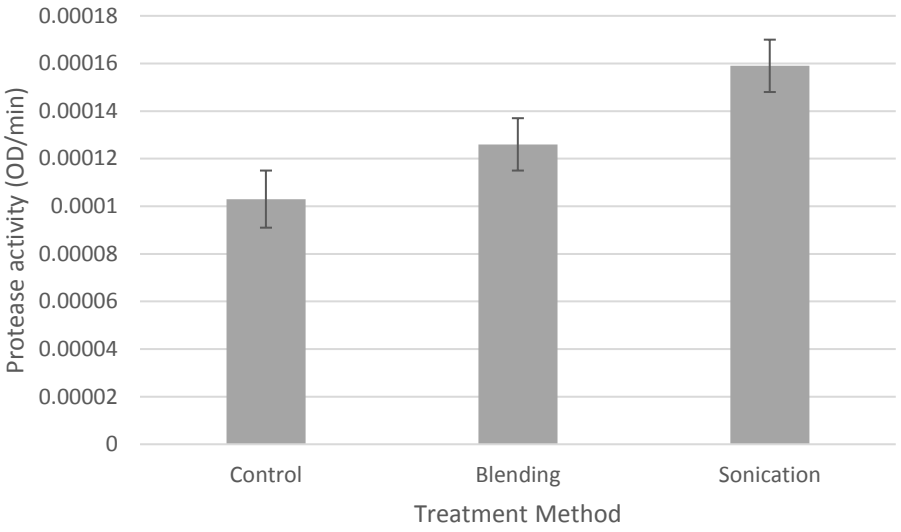

Figure 3.12 Least squares means for the effect of treatment on protease activity.

The protease activity of cow 1 was significantly higher than the activity of cow 2 (P < 0.0001). The least squares means for the effect of cow on protease activity can be seen in Figure 3.13. There was also a significant cow by treatment interaction on protease activity (P = 0.0023). The least squares means for the effect of cow by treatment interaction on protease activity can be seen in Figure 3.14.

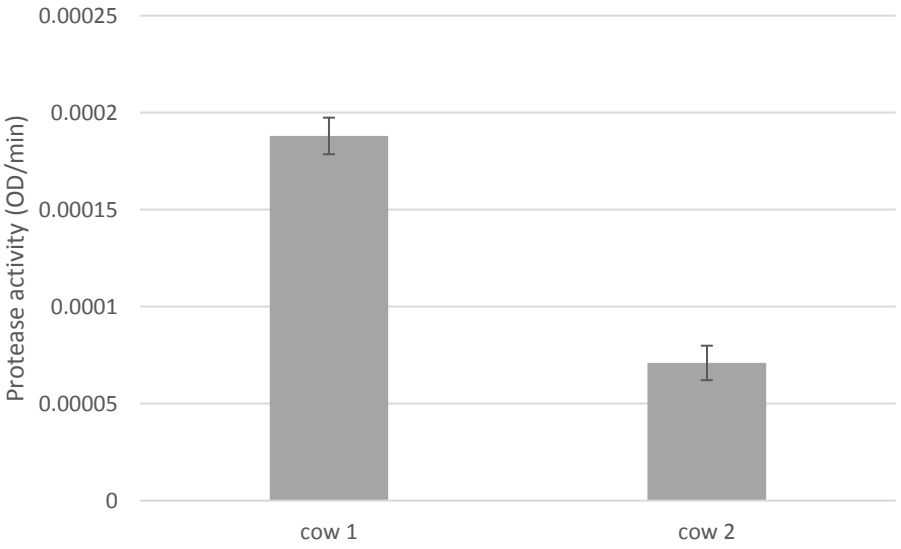

Figure 3.13 Least squares means for the effect of cow on protease activity.

The protease activity of cow 1 was significantly higher than cow 2 for control (P = 0.0055), blending (P < 0.0001) and sonication (P < 0.0001).

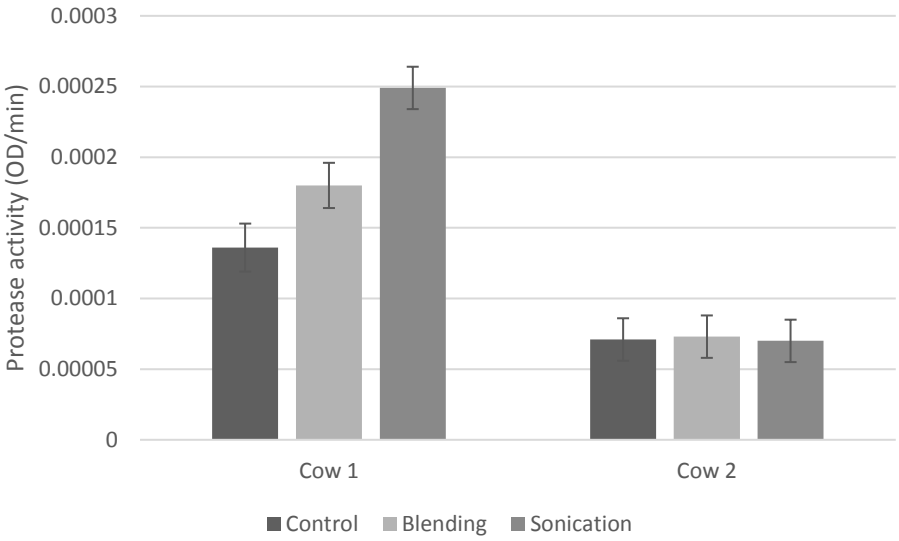

Figure 3.14 Least squares means for the effect of cow by treatment interaction on protease activity.

3.4.6 In vitro starch digestibility

The effect of treatment method on ivSd was not significant (P = 0.4408). Blending was unsuccessful in liberating amylolytic microorganisms from the particulate fraction of the rumen fluid (P = 0.4408). However, there was an effect of cow on ivSd (P = 0.0004). The least squares means for the effect of cow on ivSd can be seen in Figure 3.15. The ivSd of cow 1 was significantly higher than the ivSd of cow 2 (P = 0.0004). There was no effect of cow by treatment interaction on ivSd (P = 0.3317).

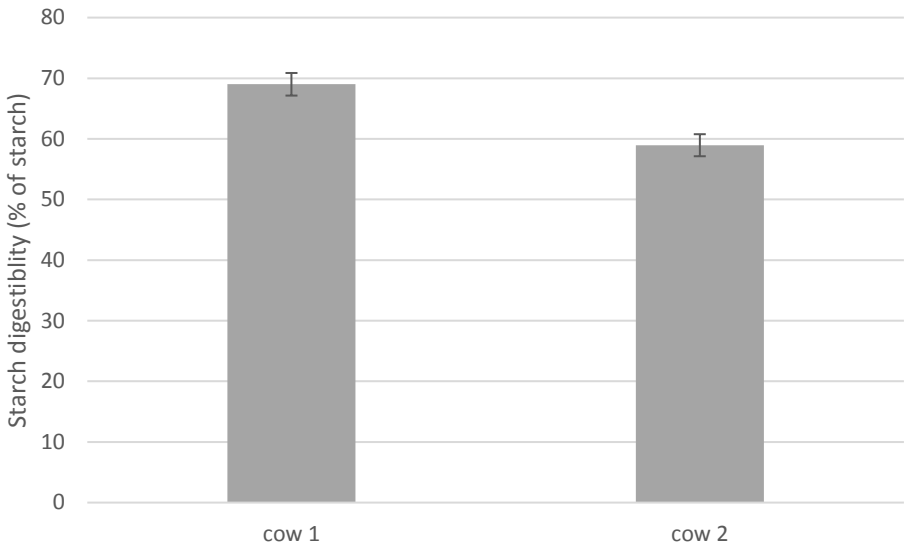

Figure 3.15 Least squares means for the effect of cow on *in vitro* starch digestibility.

3.4.7 In vitro neutral detergent fibre digestibility

There was a significant effect of treatment on NDFd (P = 0.0403). The least squares means for the effect of treatment on NDFd can be seen in Figure 3.16. The NDFd for control was significantly higher than the NDFd of the blended rumen fluid (P = 0.0403).

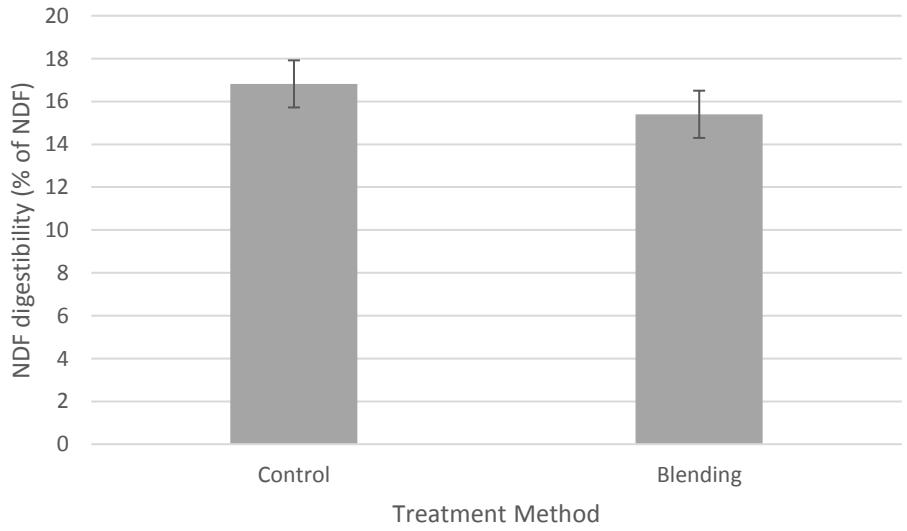

Figure 3.16 The effect of treatment on *in vitro* NDFd.

The NDFd for cow 1 was significantly higher than the digestibility of cow 2 (P = 0.0133). The least squares means for the effect of cow on digestibility can be seen in Figure 3.17. The least

squares means over time for NDFd can be seen in Figure 3.18. As expected the effect of time on NDFd was significant ($P < 0.0001$) when pooling cows, resulting in increased NDFd with longer incubations. There was no significant effect of cow by treatment ($P = 0.9098$), hour by treatment ($P = 0.9801$) or cow by hour ($P = 0.4958$) interaction on NDFd.

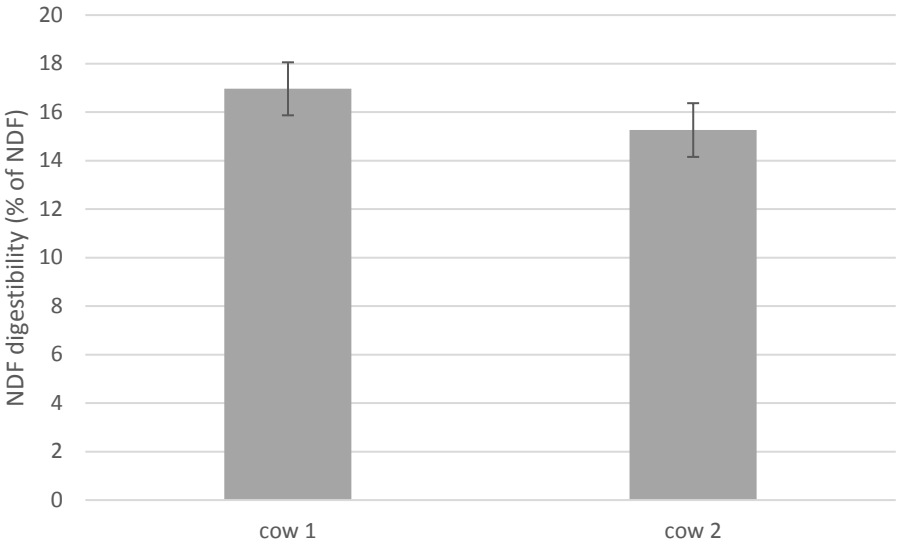

Figure 3.17 Least squares means for the effect of cow on NDFd.

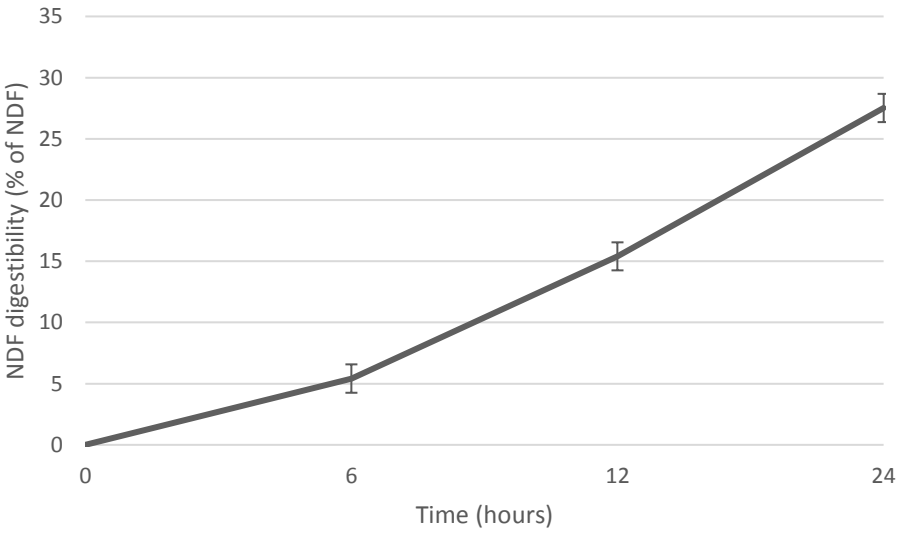

Figure 3.18 Least squares means for NDFd over time.

3.4.8 In vitro nitrogen digestibility

There was a significant effect of treatment on Nd (P = 0.0168). The least squares means for the effect of treatment on Nd can be seen in Figure 3.19. The Nd for the control was significantly higher than the digestibility of the blended rumen fluid (P = 0.0168). There was no significant difference in Nd between the cows (P = 0.2522). The effect of cow by treatment interaction was also non-significant (P = 0.2415).

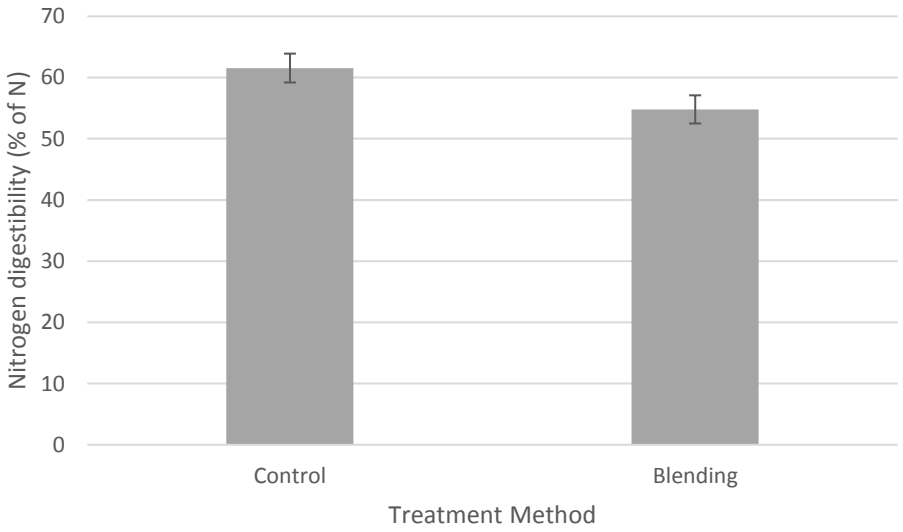

Figure 3.19 Least squares means for the effect of treatment on Nd.

3.5 DISCUSSION

The objective of this experiment was to determine the effect of processing of rumen fluid on enzymatic activity and *in vitro* digestibility. Processing of rumen fluid was successful in liberating amylolytic enzymes associated with the particle fraction, without favouring one method. The diet (TMR) could have contributed to the results as Nozière and Michalet-Doreau (1997) found that an increase in the level of barley in the diet, that is rapidly available (compared to maize with a resistant endosperm), increased the amylase activity of solid-associated ruminal microorganisms. However, Martínez *et al*. (2002) have shown that there is a significantly higher concentration of amylase activity associated with the particulate fraction compared to the bacteria and liquid fraction of the rumen

content isolated from sheep fed a lucerne and maize diet. Results obtained by Hristov *et al*. (1999) showed that the amylase activity of sonicated rumen fluid was significantly higher than the activity of untreated rumen fluid. These results were unaffected by the diet of the cows (high-forage vs high-grain). Lee *et al*. (2002) obtained contrasting results where sonication of rumen fluid sampled from bulls on a lucerne based diet failed to increase the amylase activity compared to untreated rumen fluid. The contrasting results could be attributed to methodological differences, as the whole rumen content samples of Lee *et al*. (2002) were first incubated at 39°C for 60 min under anaerobic conditions to separate the small and large particles by sedimentation. Both particle fractions were then recombined and suspended in a sodium phosphate buffer. They then proceeded to remove the non-adherent microbial population from both fractions using a procedure described by Merry and McAllan (1983). The enzymatic activity associated with the liquid fraction of the rumen was then separated from the enzymes associated with the particulate matter by centrifugation. Thereafter, the pellet obtained by centrifuging was sonicated (anaerobic, on ice, 2min, 3-second intervals) to obtain the cell-bound enzyme fraction. Hristov *et al*. (1999) had three rumen content sample types, namely (1) unfiltered rumen content, (2) a combination of filtered rumen content and the filtrate of solid particles combined with a phosphate buffer containing dithiothreitol, and (3) a combination of filtered rumen content and the blended (high, 30 s) filtrate of solid particles combined with a phosphate buffer containing dithiothreitol. Samples were sonicated (three times 30 s, with 30 s intervals) and the enzymatic activity of the supernatant was determined. Their results showed no interaction between sonication and the sample type ($P < 0.05$).

The amylolytic activity of the control was almost double for cow 1 compared to cow 2. However, the amylolytic activity after blending and sonication did not differ between the cows. This would suggest that although there was a difference in the amylolytic activity in the liquid fraction of the rumen fluid between the two cows as demonstrated by the difference in the control samples ($P < 0.0001$), processing was able to reduce that difference by freeing of amylolytic enzymes associated with the particulate fraction consistently for both cows. In other words, the amount of particle-

associated amylolytic enzymes were similar between the two cows, but the difference observed in the activity of the liquid-associated (free) enzymes was probably attributed to a difference in the rumen dilution rate between the two cows. The two cows had an *ad libitum* supply of water, and this might have caused a difference in the concentration of the microorganisms present and consequently a difference in the concentration of free enzymes.

Processing of rumen fluid was successful in liberating lichenase enzymes associated with the particulate fraction, by either blending or sonication, without favouring one method. The interactions observed for lichenase activity would suggest that the initial free lichenase enzymes within the rumen fluid differed between the two cows with the activity of free lichenase being higher in that of cow 1. Seeing as cow 2 had a lower initial lichenase activity, and the activity for the blended rumen fluid was higher than that of cow 1, it could be suggested that cow 2 had more lichenase enzymes associated with the particulate fraction. Even though the lichenase activity of the sonicated samples did not differ significantly between the two cows, there is a bigger difference between cow 2's control sample and sonicated sample compared to the difference between cow 1's control sample and sonicated sample (as seen in Figure 3.5).

Lee *et al*. (2002) reported that the CMC degrading enzyme, β-D-endoglucanase present in the rumen of bulls on a lucerne based diet, was mainly bound to microbial cells. Similar to the results obtained in this study, Lee *et al*. (2002) found that sonicated rumen fluid had a higher endoglucanase activity compared to the activity associated with the whole rumen content (liquid and particles), rumen liquor or feed particles. Hristov *et al*. (1999) also reported that the endoglucanase activity of sonicated rumen fluid was higher than the activity of untreated rumen fluid. Williams and Strachan (1984) reported that the enzymes produced by bacteria and protozoa responsible for CMC degradation displayed the highest activity in the particle-associated adherent population. Therefore, processing of rumen fluid, by either blending or sonication without favouring one method, was therefore successful in liberating endoglucanase enzymes associated with the particulate fraction in the present study. The lack of significant difference for the blended samples would suggest that both cows had a similar

amount of endoglucanase enzymes associated with the particle fraction. However, cow 1 had a greater amount of cell-bound enzymes and liquid-associated enzymes as depicted by the difference observed between the sonicated and control samples, respectively. The greater amount of cell-bound endoglucanase enzymes liberated by Cow 1, could be because of a greater amount of endoglucanase producing microorganisms present in the rumen of cow 1 compared to cow 2. Additionally, the difference observed in the activity of the liquid-associated enzymes was probably attributed to a difference in the rumen dilution rate between the two cows. These differences observed could be the result of a difference in water and dry matter intake between the two cows as the cows differed in their average daily milk production. However, this is not possible to confirm without monitoring the feeding and drinking activity.

Processing of rumen fluid, by either blending or sonication, was successful in liberating xylanase enzymes associated with the particulate fraction, without favouring one method. This was also demonstrated by Michalet-Doreau *et al*. (2001) who showed that the xylanase activity, of sheep fed a lucerne diet, was solidly associated with the particulate fraction. Similar results were reported by Lee *et al*. (2002) who showed that sonicated rumen fluid sampled from bulls on a hay based diet had a higher xylanase activity compared to the activity associated with the whole rumen content (liquid and particles), rumen liquor or feed particles. Hristov *et al*. (1999) also reported that the xylanase activity of sonicated rumen fluid was significantly higher than the activity of untreated rumen fluid. The xylanase activity of Cow 1 remained significantly higher than the activity of cow 2, even with the treated rumen fluid. Blending and sonication were, therefore, unable to reduce the gap in xylanase activity between cow 1 and cow 2.

Lee *et al*. (2002) and Kohn and Allen (1995) showed that sonication and blending failed to produce a higher protease activity compared to the untreated rumen fluid. Lee *et al*. (2002) reported that most of the protease activity in the rumen of bulls fed a lucerne based diet was associated with the liquid fraction and the microbial cells. This could explain why there was no significant difference in protease activity between the control and blended rumen fluid (P = 0.1515) in the present study,

because there could have already been a high concentration of enzymes associated with the liquid fraction of the rumen content. The higher protease activity displayed by sonication could be attributed to the extraction of intracellular enzymes by cell lysis while blending was not successful in liberating cell-associated enzymes. The protease activity of Cow 1 remained significantly higher than the activity of cow 2, even with the treated rumen fluid. Blending and sonication were, therefore, unable to reduce the gap in xylanase activity between cow 1 and cow 2.

Blending was unable to improve ivSd, although the origin of the rumen fluid did affect the results. The fact that blending did improve amylolytic activity in the enzymatic assay suggests that the amylolytic microbes responded differently to the treatment compared to the enzymes.

The NDFd and Nd for control were significantly higher than the digestibilities of the blended rumen fluid. Similar results have been reported by Craig *et al*. (1984) with regard to NDFd for strained rumen fluid that was blended without the addition of particle-associated organisms. The decrease in *in vitro* digestibility observed with the blended rumen fluid could be attributed to cell lysis of NDF and protein-digesting microorganisms. This is of importance as blending is frequently used and suggested in *in vitro* digestibility studies for the extraction of adherent microorganisms (Getachew *et al*., 2000; McAllister and Sultana, 2011; Kisidayova and Jalc, 2013).

For the effect of cow, cow 1 consistently (except for lichenase activity) displayed higher enzymatic activities and *in vitro* digestibility values compared to cow 2. As previously mentioned, Weimer *et al*. (2010) and Welkie *et al*. (2010) has shown that the bacterial community composition is distinctive to individual cows. Additionally, the difference observed in enzymatic activity might be due to a difference in the daily intake between the cows since there is a big difference in their number of days in milk and their average daily milk production. A difference in intake would have consequences on the measured enzymatic activity and *in vitro* digestibility values even if the cows were fasted, likely due to different passage and dilution rates. Additionally, the rumen content is in continuous movement because of ruminal contractions, and these movements are also affected by the amount of intake. The fact that rumen fluid was only sampled from the ventral rumen and was not a

mixture of rumen content from different sampling sites, could also have attributed to the results observed. However, this is impossible to confirm without knowing the daily dry matter intakes and the respective feeding behaviour.

3.6 CONCLUSION

Both blending and sonication had the ability to consistently release more amylase, lichenase, endoglucanase and xylanase enzymes. Sonication is a more complex treatment and therefore blending is an adequate alternative when enzymatic activity of rumen liquor needs to be quantified. However, blending was not able to release more protease. Therefore, based on our results, sonication cannot be replaced by blending for the extraction of cell-associated protease enzymes. For rumen *in vitro* digestibility, blending was not successful in liberating amylolytic microorganism or enzymes associated with the particulate fraction and might have negatively affected the microbial population responsible for fibre and protein digestion. Therefore, as opposed to what is sometimes suggested, blending rumen fluid before an *in vitro* trial does not necessarily result in higher digestibility values and reduced lag (i.e. higher microbial activity) and could result in opposite results.

3.7 REFERENCES

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CHAPTER 4

THE DIURNAL PATTERNS OF RUMINAL ENZYMATIC ACTIVITY AND *IN VITRO* DIGESTIBILITY OF STARCH, NDF AND NITROGEN

4.1 ABSTRACT

When performing ruminal *in vitro* studies, not enough attention is given to the time of day when rumen fluid is sampled, thereby not allowing for variations caused by diurnal patterns and the effect it might have on starch, neutral detergent fibre (NDF) and protein digestibility. The objective of this study was to determine if diurnal patterns in starch, NDF and protein digestibilities and amylolytic, fibrolytic and proteolytic activities exist in dairy cows. Rumen fluid was collected before the morning feeding and subsequently every 4-hours for a 24-hour period. Two of the cows were restricted from feed for 8-hours overnight, and the other two continued to receive their feed *ad libitum*, to isolate but also quantify the effect of a possible different feeding behaviour at night. After two runs the cows were crossed over between night feeding treatments. The rumen fluid was analysed for amylase, lichenase, endoglucanase, xylanase and protease activity and *in vitro* starch, NDF and nitrogen digestibility. Under natural grazing conditions, cows display a diurnal feeding pattern where most of the feeding activity occurs during the day, especially at sunset and sunrise. However, the diurnal feeding pattern of dairy cows in a free-stall housing setup tends to be influenced by milking, feed push-up and the time of feed delivery. Patterns in activity observed for amylase, lichenase, endoglucanase and xylanase were most likely a result of feeding behaviour adapted to the time of feed delivery and milking. However, protease activity was unaffected by either feeding treatment or possible feeding behaviour, although when fitted to a cosine function it did display a daily pattern that was sensitive to the availability of feed overnight. The patterns displayed by *in vitro* digestibility results of starch, NDF and nitrogen, across the various fluid collection time points, were much more variable than expected, probably because various factors affect the final result, most likely related not

only to the dynamic complexity of the rumen but also to the microenvironment within each flask, the human factor and the whole *in vitro* procedure. Such a variation would result in important consequences when computing rates of digestion, especially for starch, to be used for rationing software systems like the CNCPS. When fitted to a cosine function all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed, although the parameters responded differently to the feeding treatment.

4.2 INTRODUCTION

Circadian rhythms have been observed in many organisms including animals, plants and bacteria, allowing them to adapt their physiological requirements in a pre-emptive manner to the time of day (Brown and Schibler, 1999). A circadian rhythm can be defined as any internal process related to a living organism that displays an entrainable oscillation over a 24 h period. Endogenous clocks allow organisms to synchronise their physiological and behavioural activities with changes in the exogenous environment (Harvatine, 2012). In animals, the wake and sleep pattern is the most apparent activity regulated by circadian rhythms (Takahashi *et al*., 2008). Circadian patterns will continue when environmental signals are absent, as they are controlled by time-keeping mechanisms in the peripheral tissues and central nervous system (Niu *et al*., 2014). In mammals, the brain houses the master timekeeper in the hypothalamic suprachiasmatic nucleus (SCN; Brown and Schibler, 1999; Takahashi *et al*., 2008). The circadian clock located in the SCN is entrainable by light (Brown and Schibler, 1999) and is reset daily by the photoperiod when light signals perceived by the retinohypothalamic tract are transmitted to the SCN (Balsalobre *et al*., 2000). Pacemakers in peripheral tissues are entrainable by environmental stimulants including temperature cycles, hormonal signals and the timing of feed availability (Balsalobre *et al*., 2000; Damiola *et al*., 2000; Harvatine, 2012). According to Takahashi *et al*. (2008) in humans the master timekeeper and pacemakers in peripheral tissues can be desynchronised by the timing of food intake, increasing the occurrence of various disorders (Bass and Takahashi, 2010). Harvatine (2012) suggested that certain

management strategies employed by dairy farmers can desynchronise the master timekeeper and mammary timekeepers and possibly desynchronise absorption of nutrients and milk production, consequently decreasing milk yield. Numerous physiological activities in cows have been reported in the literature (Lefcourt *et al*., 1999; Piccione *et al*., 2007; Giannetto and Piccione, 2009) to follow a circadian pattern, including glucose, urea, non-esterified fatty acids (NEFA), total lipids, total cholesterol, haemoglobin, creatinine, magnesium, phosphorus, respiratory rate and rectal temperature. Milk production in dairy cows has also been shown to follow a circadian pattern that is sensitive to the timing of feed intake (Rottman *et al*., 2014). Under natural grazing conditions, cows display a diurnal feeding pattern where most of the feeding activity occurs during the day, especially at sunset and sunrise (DeVries, 2013). However, the diurnal feeding pattern of dairy cows in a free-stall housing setup tends to be influenced by milking, feed push-up and the time of feed delivery (DeVries *et al*., 2003). The feeding time of dairy cows has the ability to retrain the daily rhythm of feeding, core body temperature, faecal neutral detergent fibre (NDF) and indigestible neutral detergent fibre (iNDF) concentrations, lying behaviour and plasma blood urea nitrogen, insulin and glucose concentrations (Niu *et al*., 2014). Robinson *et al*. (1997, 2002) showed that the time of feeding protein supplements to dairy cows affected the diurnal patterns of ruminal fermentation, volatile fatty acids (VFA), and amino acids. Blackburn and Hobson (1960) also reported that VFA's were affected by the time of feeding. Additionally, Russell *et al*. (1981) showed that the most significant increase in ammonia was observed 1 to 3-hours after feeding. In addition to protein metabolites, Cone *et al*. (1989) and Fickett and Allen (2002) showed that the extent of *in vitro* starch digestibility was also affected by feeding time. Fickett and Allen (2002) attributed the variation in digestibility rates to the difference in enzymatic activity of rumen fluid collected before and after feeding. There is, therefore, a reason to speculate if diurnal patterns for enzymatic activity might not also exist as bacterial numbers have already been shown to be influenced by the time of feeding (Bryant and Robinson, 1968). When performing ruminal *in vitro* studies, not enough attention is given to the time of day when rumen fluid is sampled. Thereby not allowing for variations caused by diurnal

patterns and the effect it might have on starch, NDF and protein digestibility. The results of these studies are used to characterise feeds, to determine the amount of feed needed in the diet of the cow to meet its requirements and especially for the determination of starch, NDF and protein requirements. Additionally, often the results between *in vitro* studies are compared without taking the time of sampling or the feeding time into account as a source of variability. The objective of this study was to determine if diurnal patterns in starch, NDF and protein digestibility and amylolytic, fibrolytic and proteolytic activity exists in dairy cows.

4.3 MATERIALS AND METHODS

Rumen fluid was sampled by hand from four rumen fistulated lactating Holstein cows before the morning feeding at the Welgevallen experimental farm located at Stellenbosch University. The fluid was obtained from the ventral area of the rumen of the four cows (515.75 ± 89.07 DIM, 735.67 ± 87.64 kg, 5.42 ± 2.33 years old, 17.12 L ± 10.40 daily milk production, mean ± SD). Based on the results of the previous experiment (Chapter 3), the fluid of each cow was treated separately (i.e. not mixed) to isolate the effect of cow and to increase the power of the study (i.e. to reduce the residual error term). Subsequent collections were every 4-hours for a 24-hour period (7h30, 11h30, 15h30, 19h30, 23h30 and 03h30). The cows were milked at 06h00 and 16h00. Feed delivery was at 07h30 and 16h00. The first collection took place before the morning feeding and right after cows were milked. The cows received a total mixed ration *ad libitum*, with maize as the main source of starch and lucerne and wheat straw as the main sources of NDF. Two of the cows were restricted from feed for 8-hours overnight, and the other two continued to receive their feed *ad libitum*, to isolate but also quantify a possible different feeding behaviour at night. After two runs the cows were crossed over and the cows previously receiving their feed *ad libitum* overnight were then fasted for the next two runs. The cows were allowed five days to adapt to the fasting before the commencement of the study and after the crossover from fasting to *ad libitum* and vice versa. The objective of the adaptation was to adapt the animal and the rumen microorganisms to the lack of feed overnight, and since there was

no change in the diet itself, it is strongly believed that five days were sufficient. The adaptation period is supported by recent research about rumen changes and adaptation (Grant *et al*., 2015) and the fact that complete rumen turnover in lactating cows usually occurs in no more than 48-hours (Van Soest, 1994). All the procedures used in this study were approved by the Research Ethics Committee for Animal Care and Use of Stellenbosch University (SU-ACUD14-00052).

4.3.1 Determination of enzymatic activities

Rumen fluid samples for the enzymatic trial were placed on ice at sampling to slow down microbial activity. The rumen fluid was filtered through four layers of cheesecloth, glass wool and two layers of °200 µm porosity mesh. The samples were centrifuged at 16 000 × g for 15-minutes and only the supernatant was used for enzymatic analysis. Enzymatic assays followed immediately.

Enzymatic activity of amylase, endoglucanase, lichenase and xylanase were assessed colourimetrically by measuring the amount of reducing sugars released using dinitrosalicylic acid (DNS; Miller, 1959). Amylolytic activity was determined using 0.2% (w/v) cooked maize starch (S4126, Sigma-Aldrich, USA) resuspended in 0.05 M citrate buffer (pH 6.0). Endoglucanase activity was determined using 0.1% (w/v) lichenan (L6133, Sigma-Aldrich, USA) and 1% (w/v) carboxymethyl cellulose (CMC; C5678, Sigma-Aldrich, USA). Activity measured using CMC and lichenan as substrates will be referred to as endoglucanase and lichenase activity, respectively. Endoxylanase activity was determined using 1% (w/v) Beechwood xylan (X4252, Sigma-Aldrich, USA) resuspended in the same buffer. Enzymatic activities were determined by measuring the amount of reducing sugars released from substrates during 15-minutes at 39°C with 450 µl of substrate and 50 µl of enzyme sample. The mixture was boiled with 750 µl DNS for 15-minutes to stop the reaction. Spectrophotometry was used to quantify the reducing sugars at 540 nm. One unit was determined as 1 µmol reducing sugar liberated per minute using glucose or xylose as standard.

Protease activity was assessed colourimetrically by measuring the amount of azo dye released from 2% azocasein (A-2765, Sigma-Aldrich, USA) in a 0.1 M sodium phosphate buffer (pH 6.8) as

per the procedure by de Vyver *et al*. (2004). Except protease activity was defined as optical density per minute and was determined by subtracting the optical density of the blank from the optical density of the test sample and dividing that value by the total incubation time (in minutes).

4.3.2 In vitro fermentations

Rumen fluids were sampled to determine *in vitro* starch digestibility (ivSd), neutral detergent fibre digestibility (NDFd) and nitrogen digestibility (Nd) using maize, oat hay and soya oilcake, respectively. All the substrate samples were milled through a 1 mm screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ, USA). Before milling, oat hay was first oven dried at 60°C for 48-hours. All the substrates were analysed for dry matter (DM; AOAC, 2002, Method 934.01). Residual starch was analysed as described by Hall (2000). Residual NDF was analysed as described by Raffrenato and Van Amburgh (2011), with the addition of amylase and sodium sulphite anhydrous. Nitrogen digestibility was quantified as per the rumen digestibility procedure by Ross *et al*. (2013) and crude protein (CP), used for the determination of Nd, was determined using a Nitrogen Gas Analyzer FP528 (LECO Africa Pty Ltd, Kempton Park).

Rumen fluid samples for the digestibility trial were placed in a pre-warmed insulated flask at collection. Rumen fluid was filtered into a pre-warmed Erlenmeyer flask through four layers of cheesecloth, glass wool and two layers of °200 µm porosity mesh. Carbon dioxide was pumped into the flasks to displace any air. Substrate samples were weighed into 125 ml Erlenmeyer flasks, and 40 ml *in vitro* buffer medium (adapted from Goering and Van Soest, 1970) was added to each sample. The flasks were then placed in a water bath (39°C) and flushed with CO_2. Flask preparation was done before rumen fluid sampling as to allow the buffer medium to reach temperature before adding the rumen fluid. Rumen fluid (10 ml) was injected into each flask using a prewarmed automatic syringe. Inoculations were always completed within 15-minutes after collection. The ivSd samples were incubated for 7-hours, which is the common time point used for rumen *in vitro* starch digestion. The ivSd at 7-hours is usually also used to estimate the rate of starch digestion (Sniffen and Ward, 2011).

Preliminary results for NDF digestibility showed that the difference between the treatments was reduced with a traditional 24-hour incubation. Therefore, the NDF incubation was performed for 12-hours to ensure differences in NDFd between the treatments (if present) were detectable. The Nd samples were incubated for 8-hours. The common time point used to estimate rumen degradable protein (RDP) is 16-hours with the objective of obtaining the maximum extent of digestion *in vitro* or *in situ*. We chose 8-hours for the *in vitro* incubation to increase the likelihood of detecting differences in Nd. All samples were incubated in duplicate, and all fermentations were completed across four runs.

4.3.3 Statistical analysis

In vitro NDF, starch and N digestibility values, amylase, endoglucanase, xylanase, lichenase and protease activities were analysed as response variables by the GLIMMIX procedure of SAS (version 9.3; SAS Institute, Inc., Cary, NC) using a factorial arrangement of *ad libitum* vs. fasting (treatment), collection time and their interactions. Cow was treated as random factor. Differences were declared significant at $P \leq 0.05$ using the least squares means and the Tukey adjustment. Statistical differences resulting in $0.05 < P \leq 0.10$ were considered tendencies. The results of treatments are reported as least squares means.

Circadian rhythm analysis of enzymatic activity and *in vitro* digestibility were conducted by fitting the linear form of a cosine function with a 24-hour period according to Bourdon *et al*. (1995) by random regression in SAS (Seltman, 1997). A zero amplitude *F*-test was performed to compare the cosine fit to a linear fit to determine the significance of the 24-hour cosine fit for each of the treatments. Phase refers to the time at which the peak of the cycle occurs, and is reported as acrophase. The 95% confidence limits were determined for both phase and amplitude (Bourdon *et al*., 1995). The treatment differences were considered significant when the difference of phase or amplitude were 1.96 times the square root of the sum of squares of the standard error values ($P < 0.05$; Knezevic, 2008). All response variables were checked for outliers and removed if respective Studentized

residuals were outside of the range ±3 (Niu *et al*., 2014). No more than two data points were removed per response variable.

4.4 RESULTS

4.4.1 Amylolytic activity

There was no significant difference between the amylolytic activities of the cows fasted the night before and the cows fed *ad libitum* ($P = 0.7756$). The time of rumen fluid collection did however significantly affect the amylolytic activity ($P < 0.0001$). The least squares means for the effect of time of collection on amylolytic activity can be seen in Figure 4.1 and the *P*-values of the differences of time of collection least squares means can be seen in Table 4.1. The highest amylolytic activity was observed at 19h30 and was significantly higher than the activity at all the other time points, when pooling the main treatment levels (fasting vs. *ad lib*) namely 03h30 ($P < 0.0001$), 07h30 ($P < 0.0001$), 11h30 ($P = 0.0223$), 15h30 ($P < 0.0001$) and 23h30 ($P < 0.0001$). The second highest activity was observed at 11h30. The lowest amylolytic activities were observed at 07h30 and 15h30.

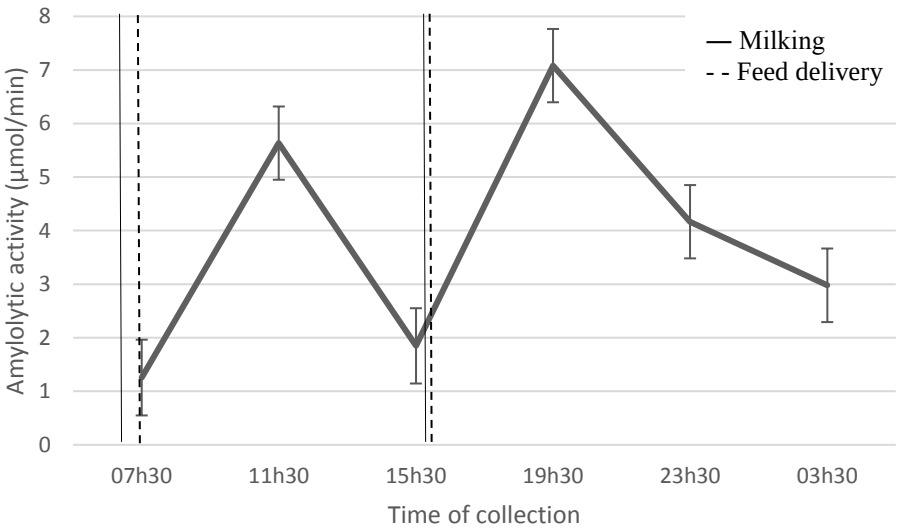

Figure 4.1 Least squares means for the effect of time of collection on amylolytic activity.

Table 4.1 Least squares means for the effect of time of collection on enzymatic activity and *in vitro* digestibility.

	Least squares means					
Parameter	07h30	11h30	15h30	19h30	23h30	03h30
Enzymatic Activity						
Amylase, µmol/min	1.2559^{e}	5.6338^{b}	1.8477^{de}	7.0810^{a}	4.1659^{c}	2.9786^{d}
Lichenase, µmol/min	1.8106^{c}	4.2369^{a}	1.9218^{bc}	4.3177^{a}	2.3703^{b}	2.1948^{bc}
Endoglucanase, µmol/min	1.5750^{b}	2.5150^{a}	1.5367^{b}	2.4287^{a}	1.6965^{b}	1.5841^{b}
Xylanase, µmol/min	2.3540^{c}	4.7716^{a}	2.5357^{c}	5.3492^{a}	3.2959^{b}	2.7237^{bc}
In vitro digestibility						
Starch, % of starch	33.93^{cd}	39.36^{ab}	35.82^{bc}	30.08^{d}	43.41^{a}	40.56^{a}
NDF, % of NDF	14.41^{bc}	16.07^{a}	14.72^{ab}	12.79^{d}	13.15^{cd}	12.09^{d}
Nitrogen, % of N	48.02^{a}	42.48^{b}	40.14^{bc}	40.94^{b}	36.26^{c}	39.21^{bc}

$^{a\text{-}e}$ Means within a row not sharing a superscript differ ($P < 0.05$).

The interaction between treatment (*ad libitum* vs fasting overnight) and the time of collection tended to influence amylolytic activity ($P = 0.0648$). The least squares means for the effect of the interaction between treatment and the time of collection can be seen in Figure 4.2. There was no significant difference between the amylolytic activity for the fasted and *ad libitum* fed cows at 03h30 ($P = 0.6283$), 07h30 ($P = 0.6060$), 11h30 ($P = 0.3533$), 15h30 ($P = 0.9840$) or 19h30 ($P = 0.5926$). However, at 23h30 the amylolytic activity of the cows fasted overnight tended to be lower than the activity of the cows that continued to receive their feed *ad libitum* ($P = 0.0714$).

4.4.2 Lichenase activity

There was no significant difference between the lichenase activities of the cows fasted the night before and the cows fed *ad libitum* ($P = 0.2255$). However, there was a significant effect of the time of rumen fluid collection on lichenase activity ($P < 0.0001$). The least squares means for the effect of the time of collection on lichenase activity can be seen in Figure 4.3 and Table 4.1.

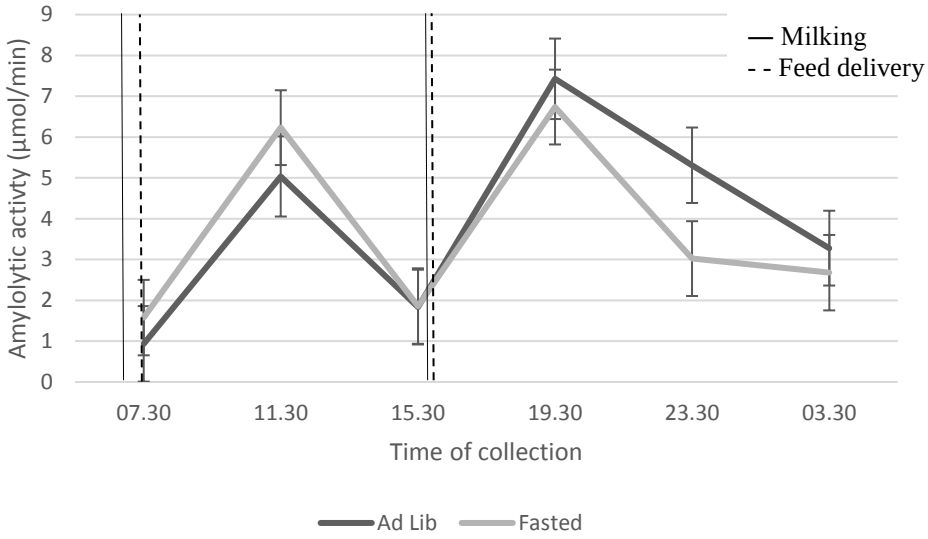

Figure 4.2 The effect of *ad libitum* or fasted treatment by time of collection interaction on amylolytic activity.

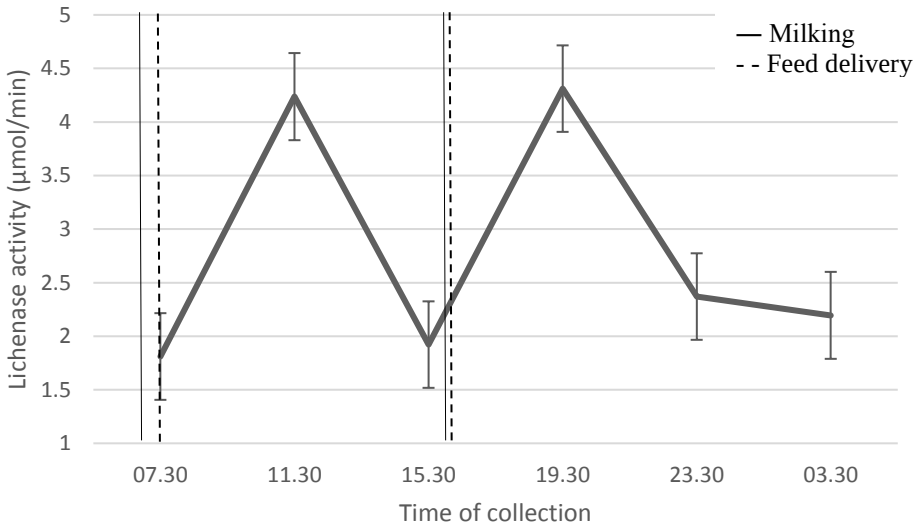

Figure 4.3 Least squares means of the effect of time of collection on lichenase activity.

The highest activity was observed at 11h30 and 19h30, and there was no significant difference between the two time points ($P = 0.7785$). The activity for the rumen fluid collected at 19h30 was significantly higher than for the rumen fluid collected at 03h30 ($P < 0.0001$), 07h30 ($P < 0.0001$), 15h30 ($P < 0.0001$) and 23h30 ($P < 0.0001$). There was no significant difference between the

lichenase activity between rumen fluid collected at 07h30 and 15h30 (P = 0.6720). Additionally, 07h30 and 15h30 also displayed the lowest activity. The interaction between the treatment and the time of rumen fluid collection was non-significant (P = 0.3969).

4.4.3 Endoglucanase activity

There was no significant difference in endoglucanase activity between the cows fed *ad libitum* over the overnight period and the cows fasted (P = 0.8573). The time of rumen fluid collection did, however, have a significant effect on the endoglucanase activity (P < 0.0001). The least squares means for the effect of the time of rumen fluid collection on endoglucanase activity can be seen in Figure 4.4 and Table 4.1. The highest endoglucanase activities were observed at 11h30 and 19h30 with no significant difference between these two time points (P = 0.5140). The lowest endoglucanase activities were observed at 03h30, 07h30, 15h30 and 23h30. There was no significant difference in activity for the rumen fluid collected at 03h30, 07h30, 15h30 and 23h30 (see Figure 4.1). Additionally, there was no interaction between the treatment and the time of collection on endoglucanase activity (P = 0.2913).

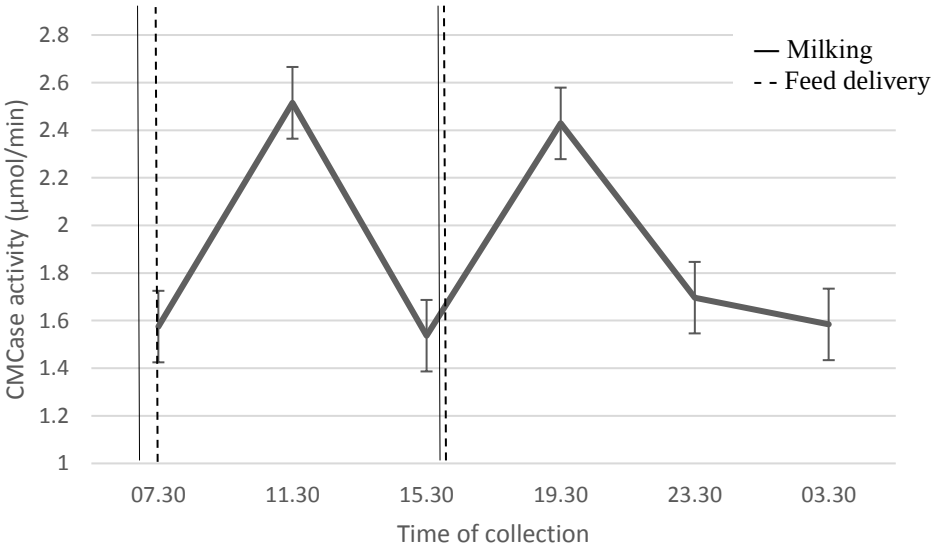

Figure 4.4 Least squares means for the effect of time of collection on endoglucanase activity.

4.4.4 Xylanase activity

There was no significant difference in xylanase activity between the cows fed *ad libitum* over the overnight period and the cows fasted (P = 0.0.7154). There was a significant effect, however, of the time of rumen fluid collection on the xylanase activity ($P < 0.0001$). The least squares means for the effect of time of rumen fluid collection on xylanase activity can be seen in Figure 4.5 and Table 4.1. The highest activity was observed at 11h30 and 19h30. There was a tendency for the xylanase activity for the rumen fluid collected at 19h30 to be higher than the activity observed at 11h30 (P = 0.0964). The xylanase activity of the rumen fluid collected at 19h30 was significantly higher than the activities observed at 03h30 ($P < 0.0001$), 07h30 ($P < 0.0001$), 15h30 ($P < 0.0001$) and 23h30 ($P <$ 0.0001). Similarly, the xylanase activity of the rumen fluid collected at 11h30 was significantly higher than the activities observed at 03h30 ($P < 0.0001$), 07h30 ($P < 0.0001$), 15h30 ($P < 0.0001$) and 23h30 ($P < 0.0001$). The xylanase activity of rumen fluid collected at 23h30 was significantly higher than the activities observed at 07h30 (P = 0.0055) and (P = 0.0238) and tended to be higher than the activity observed at 03h30 (P = 0.0915). The xylanase activity of rumen fluid collected at 23h30 did not differ significantly from the rumen fluid collected at 03h30 (P = 0.2722) and 15h30 (P = 0.5741). Similarly, there was no significant difference observed between the rumen fluid collected at 07h30 and 15h30 (P = 0.5849).

There was a significant effect of the interaction between the hour of rumen fluid collection and the treatment (P = 0.0034). The least squares means for the effect of this interaction can be observed in Figure 4.6. While there was no significant difference between the cows fed *ad libitum* and the cows fasted overnight at 03h30 (P = 0.6259), 07h30 (P = 0.9515), 15h30 (P = 0.3195), 19h30 (P = 0.1154) and 23h30 (P = 0.5587), however, a significant difference was observed at 11h30 (P = 0.0268).

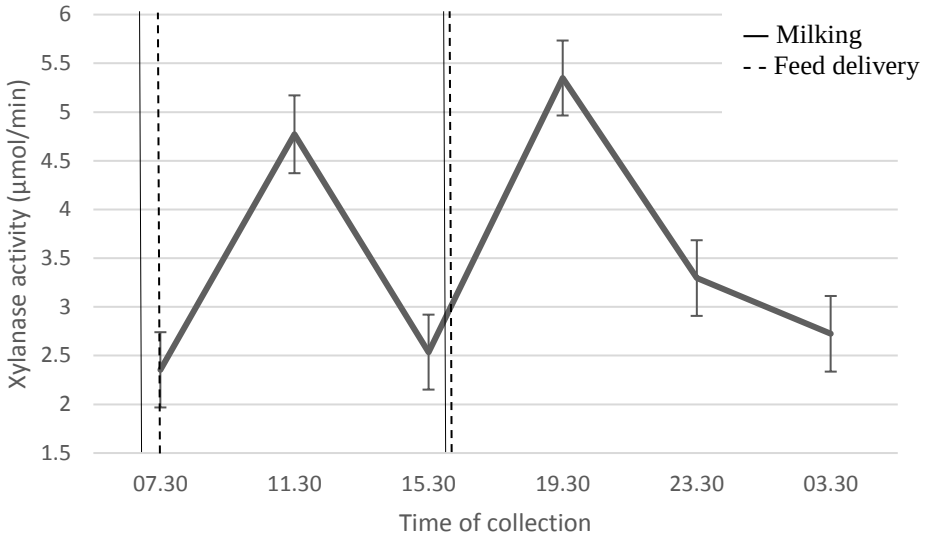

Figure 4.5 Least squares means for the effect of the time of collection on xylanase activity.

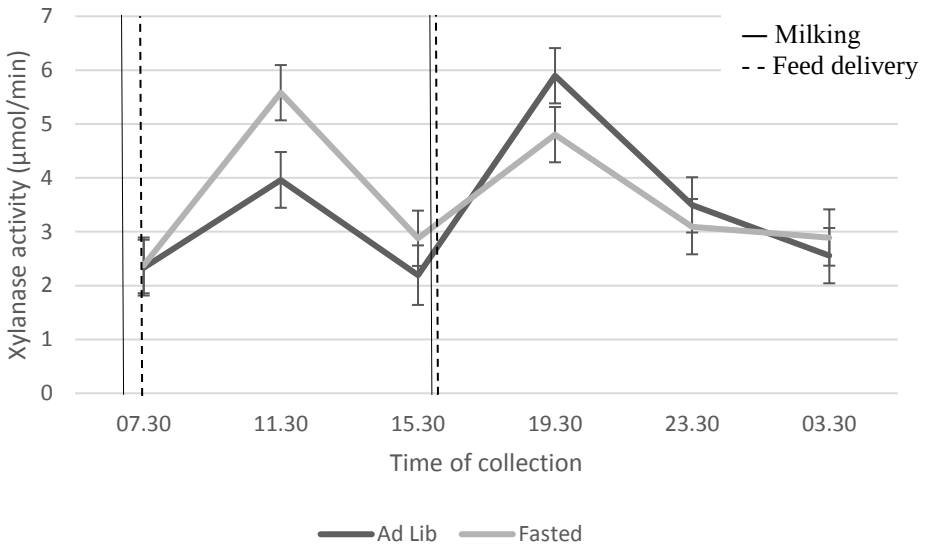

Figure 4.6 Least squares means for the effect of the interaction between the treatment and the time of rumen fluid collection on xylanase activity.

4.4.5 Protease activity

There was no significant difference in protease activity between the cows fed *ad libitum* over the overnight period and the cows fasted (P = 0.5102). Similarly, there was no significant effect of

the time of rumen fluid collection on the protease activity (P = 0.4927) or a significant effect of the interaction between the treatment and the time of collection on protease activity (P = 0.4930).

4.4.6 In vitro starch digestibility

There was no significant difference in *in vitro* starch digestibility (ivSd) between the cows receiving feed *ad libitum* overnight compared to the cows that were fasted (P = 0.9779), indicating that fasting overnight did not affect the digestibility values for the subsequent 24-hours. There was, however, a significant effect of the time of rumen fluid collection on ivSd. The least squares means for the effect of time of collection on ivSd can be seen in Figure 4.7 and Table 4.1.

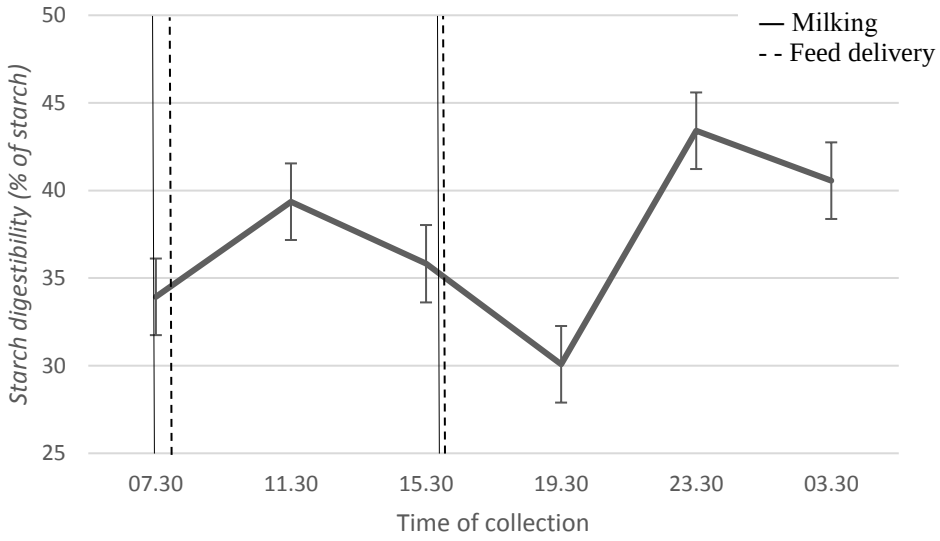

Figure 4.7 Least squares means for the effect of time of collection on *in vitro* starch digestibility.

The highest digestibility values were observed when using rumen fluid collected at 03h30, 11h30 and 23h30. Starch digestibility from 23h30 did not significantly differ from the ivSd observed at 03h30 (P = 0.2273), but the rumen fluid collected at 23h30 displayed a tendency to produce a higher ivSd than the rumen fluid collected at 11h30 (P = 0.0868). Starch digestibility from the rumen fluid collected at 23h30 was significantly higher than ivSd when using rumen fluid collected at 07h30 (P < 0.0001), 15h30 (P = 0.0017) and 19h30 (P < 0.0001). The lowest ivSd values were observed

when using fluid collected at 07h30 and 19h30. There was no significant effect of time of collection by treatment interaction on ivSd (P = 0.8139).

4.4.7 In vitro NDF digestibility

There was no significant difference in *in vitro* NDFd between the cows receiving feed *ad libitum* overnight compared to the cows that were fasted (P = 0.9136). There was, however, a significant effect of the time of rumen fluid collection on NDF digestibility (P < 0.0001). The least squares means of the effect of the time of collection on *in vitro* NDF digestibility can be seen in Figure 4.8 and Table 4.1.

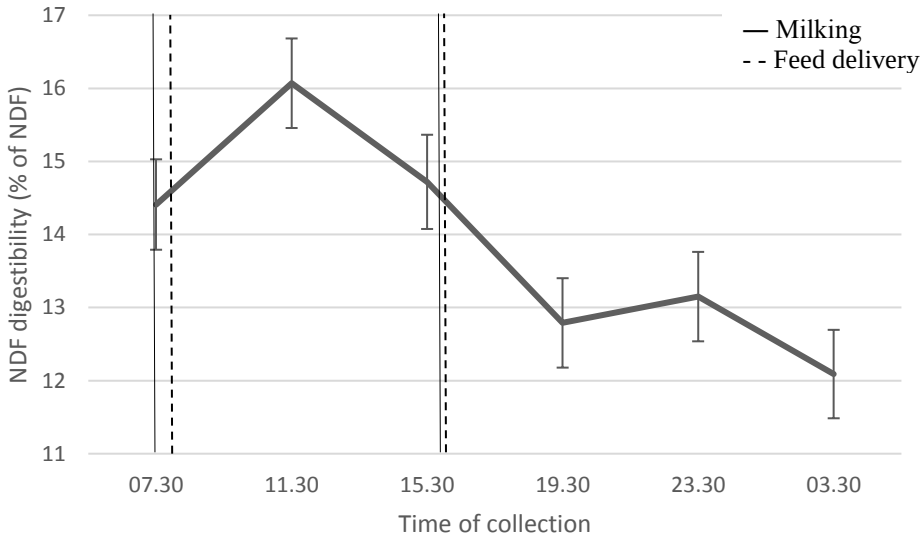

Figure 4.8 Least squares means of the effect of the time of collection on NDFd.

There was a peak in NDF digestibility at 11h30. Cell wall digestion when using rumen fluid collected at 11h30 was significantly higher than NDFd from the rumen fluid collected at 03h30 (P < 0.0001), 07h30 (P = 0.0230), 19h30 (P < 0.0001), 23h30 (P < 0.0001) and tended to be higher than the rumen fluid collected at 15h30 (P = 0.0729). The lowest NDFd values were observed from rumen fluid collect at 03h30, 19h30 and 23h30. The effect of the time of collection by treatment interaction on the extent of NDFd was non-significant (P = 0.6880).

4.4.8 In vitro nitrogen digestibility

There was no significant difference in *in vitro* nitrogen digestibility between the cows receiving feed *ad libitum* over the night period compared to the cows that were fasted ($P = 0.4836$). There was a significant effect of the time of rumen fluid collection on nitrogen digestibility ($P < 0.0001$). The least squares means for the effect of time of collection of rumen fluid on nitrogen digestion can be seen in Figure 4.9 and Table 4.1. A peak in nitrogen digestibility was observed at 07h30. Nitrogen digestion was significantly higher from fluid collected at 07h30 than the ones from 03h30 ($P < 0.0001$), 11h30 ($P = 0.0063$), 15h30 ($P = 0.0001$), 19h30 ($P = 0.0017$) and 23h30 ($P < 0.0001$). The lowest N digestibility values were observed at 03h30 and 23h30, and there was no significant difference in the N digestibility between these two time points ($P = 0.1466$). The interaction between the time of collection and treatment on the extent of Nd was non-significant ($P = 0.2005$).

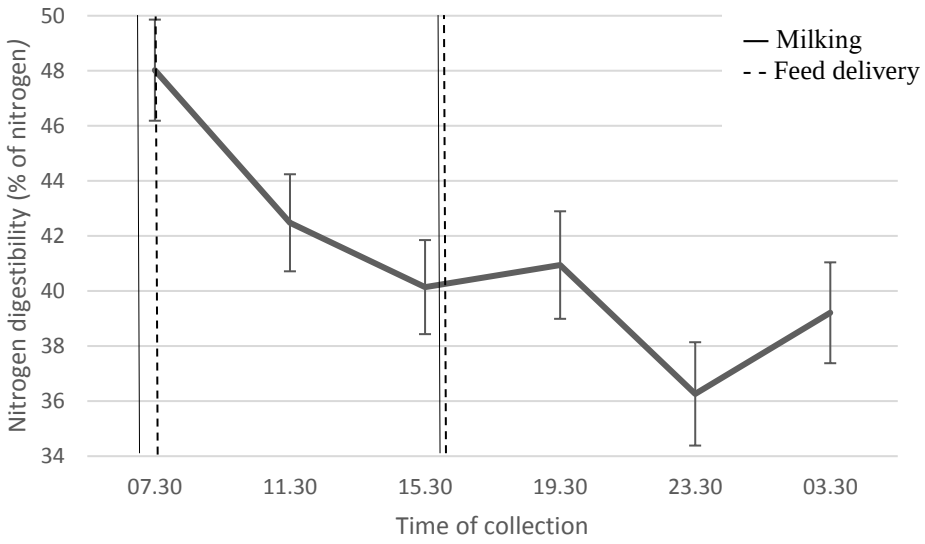

Figure 4.9 Least squares means for the effect of the time of rumen fluid collection on the *in vitro* Nd.

4.4.9 Circadian patterns

Amylase, lichenase, endoglucanase, xylanas and protease activity, and *in vitro* starch, NDF and nitrogen digestibility over the day all fit a cosine function with a 24-hour period for both treatments (Table 4.2). The daily patterns adapted to the treatment and were therefore sensitive to the availability of feed at night.

Amylolytic activity was phase advanced by 8.44-hours in the *ad libitum* fed cows compared to the fasted cows. For amylolytic activity, the amplitude of the *ad libitum* fed cows were more than 50% greater than for the fasted cows. Lichenase activity was phase advanced by 0.48-hours for the *ad libitum* cows compared to the fasted cows. For lichenase activity, the amplitude of the fasted cows were 7.4% greater than the *ad libitum* cows. Endoglucanase activity was phase delayed by 0.59-hours for the cows fed *ad libitum* compared to the fasted cows. For endoglucanase activity, the amplitude for the *ad libitum* fed cows were almost 10% greater than the fasted cows. The xylanase activity was phase advanced by 8.15-hours for the cows fed *ad libitum* compared to the fasted cows. For xylanase activity, the amplitude of the *ad libitum* fed cows was more than 15% greater than the fasted cows. The protease activity of the *ad libitum* fed cows was phase delayed by 3.02-hours compared to the fasted cows. For protease activity, the amplitude of the *ad libitum* fed cows was 32.5 times greater than the fasted cows.

Starch digestibility was phase delayed by 2.68-hours for the *ad libitum* cows compared to the fasted cows. For ivSd the amplitude of the fasted cows were almost 75% greater than the *ad libitum* fed cows. Neutral detergent fibre digestibility was phase delayed by 0.66-hours for the *ad libitum* fed cows compared to the fasted cows. For NDFd the amplitude for the fasted cows was more than 67% greater for the *ad libitum* fed cows. Nitrogen digestibility was phase advanced by 3.55-hours when fed *ad libitum* compared to the fasted cows. For Nd, the amplitude of the fasted cows was almost 85% greater than the *ad libitum* fed cows.

Table 4.2 The phase and amplitude of a cosine function with a 24h period fit to enzymatic activity and *in vitro* digestibility of cows fed *ad libitum* over the overnight period, or cows fasted.

Parameter	Treatment[1]	Phase[2] (h)	Amplitude[3]	*P*-value[4]
Enzymatic activity				
Amylase, µmol/min	Ad lib	8.740	1.931	<0.0001
	Fasted	17.175*	1.277*	<0.0001
Lichenase, µmol/min	Ad lib	16.296	0.635	<0.0001
	Fasted	16.780*	0.682*	<0.0001
Endoglucanase, µmol/min	Ad lib	15.789	0.271	<0.0001
	Fasted	15.197*	0.247*	<0.0001
Xylanase, µmol/min	Ad lib	7.324	0.954	<0.0001
	Fasted	15.473*	0.828*	<0.0001
Protease, OD/min	Ad lib	9.324	0.0065	0.0028
	Fasted	6.302*	0.0002*	0.0002
In vitro digestibility				
Starch, % of starch	Ad lib	17.139	2.254	<0.0001
	Fasted	14.459*	3.929*	<0.0001
NDF, % of NDF	Ad lib	12.501	1.289	0.0005
	Fasted	11.838*	2.161*	<0.0001
Nitrogen, % of N	Ad lib	7.134	2.877	<0.0001
	Fasted	10.685*	5.317*	<0.0001

[1]Treatments were cows fed *ad libitum* or cows fasted for approximately 8-hours over the night period.

[2]Phase refers to the time (h) where the highest value in the 24 h cycle occurs.

*Indicates *ad libitum* fed differed from fasted ($P < 0.05$).

[3]Amplitude refers to the halfway distance between the peak and the trough of a cosine function with a 24 h period. Units are shown next to the parameter.

[4]Zero amplitude test for a cosine function with a 24 h period for each treatment.

4.5 DISCUSSION

The lack of significant difference between the treatments (*ad libitum* vs fasted) indicates that fasting overnight did not affect the amylase, lichenase, endoglucanase, xylanase and protease activities or the *in vitro* digestibility of starch, NDF or nitrogen of the following 24-hours. However, except for protease, all the parameters displayed a significant effect of the time of rumen fluid collection on enzymatic activity and *in vitro* digestibility. Both peaks observed in amylase, lichenase, endoglucanase and xylanase activities were for the sampling times subsequent to feed delivery and milking. As previously mentioned, under natural grazing conditions, cows display a diurnal feeding pattern where most of the feeding activity occurs during the day, especially at sunset and sunrise (DeVries, 2013). However, the diurnal feeding pattern of dairy cows in a free-stall housing setup tends to be influenced by milking, feed push-up and the time of feed delivery (DeVries *et al*., 2003). It could be that the feeding behaviour of the cows were affected by the feed delivery and milking as seen in the study by DeVries *et al*. (2003).

The lack of significant difference between the overnight feeding treatments, time of rumen fluid collection and their interactions could indicate that the protease activity in the rumen is constant across the 24-hours and neither feeding treatments nor possible feeding behaviour affected the activity. Alternatively, the assay we used (de Vyver *et al*., 2004) was only able to detect a very low protease activity, but also highly variable activity (ranging from 0.000008 to 0.000708 OD/min in the samples analysed), resulting in high standard errors and therefore significant differences could not be detected. Extracting enzymes from the particle and cell-bound fractions by sonication could have increased the activity detected. However, the number of parameters tested throughout this trial and the time allowed for enzymatic assays between rumen fluid collection time points did not allow for sonication. It would have taken at least 30-minutes to sonicate the samples of the four cows individually for protease analysis.

In contrast to the results obtained from the amylase activity assays that produced two peaks for the rumen fluid sampled subsequent to milking and feed delivery, the ivSd assay displayed a delayed peak after the second milking and feed delivery. This difference could be attributed to the fact that enzymatic assays give an instant result of what is happening in the rumen and that time point regarding the enzymatic activity. For *in vitro* assays, rumen fluid is handled for longer as it needs to be injected in all the flasks and results are only obtained after the incubation period; indicating the capabilities of the microorganisms sampled at the given time point to adjust to the new micro-environment and to digest a substrate. In the case of ivSd, the values obtained are measured 7-hours after the enzymatic activity. If the cows adapted their feeding behaviour according to the time of feed delivery and milking, it could be suggested that after the second feed delivery there was a lag time in microbial activity that only reached a maximum 7.5-hours after feed delivery. Coincidentally, 7-hours is also the most common time point used for starch digestibility because 7-hours is believed to be the average retention time for starchy materials. Similar results, where a lag time was observed, were obtained by Cone *et al*. (1989) and showed that cows fed once a day only reached their maximum ivSd 12- and 16-hours after feeding for a low- and high-level diet (4 kg concentrate and 4 kg hay versus 8 kg concentrate and 8 kg hay) respectively. Starch digestibility values, across the various fluid collections time points, were in general more variable than expected. It is also important to point out how the most distant ivSd values (19h30 vs 23h30) had a difference of 0.1333. Such a difference would result in important consequences when computing rates of digestion (Hall, 2000) to be used for rationing software systems like the CNCPS (Van Amburgh *et al*., 2015).

Similarly, NDFd values, across the various fluid collections time points, were also more variable than expected. The most distant NDFd values (11h30 vs 03h30) had a difference of 0.0398 ($P < 0.0001$). Such a difference could result in differences when computing rates of digestion to be used for rationing software systems like the CNCPS (Van Amburgh *et al*., 2015), depending on how rates are estimated and especially when using few time points to calculate a fractional rate of digestion

(Raffrenato and Van Amburgh, 2011). We chose 12-hours as the NDFd incubation time point to attempt to highlight the differences caused by the moment of rumen fluid collection across the 24-hours. Logistical limitations did not allow us to have multiple time points from the same rumen fluid. However, we do believe that the differences would be smaller for farther time points (e.g. 24- or 30-hours), reducing the consequences of collecting rumen fluid at different time points. However, this speculation needs to be investigated further.

The decrease in digestibility observed in *in vitro* starch and NDF digestibility after the second feed delivery and for N after the first feed delivery could be as a result of a decrease in bacterial numbers. Leedle *et al*. (1982) reported a decrease in the viable bacterial numbers after feeding. Additionally, Bryant and Robinson (1968) found that bacterial numbers were at their lowest one hour after feeding. Bryant and Robinson (1968) attributed the decrease in bacteria numbers after feeding to the dilution of rumen contents by water, feed and saliva. However, Leedle *et al*. (1982) attributed the loss in viable bacterial numbers to a rapid change in the ruminal environment after feeding. Leedle *et al*. (1982) suggested that some ruminal bacteria could be adapting to the changed ruminal environment after feeding by participating in substrate-accelerated death as described by Postgate and Hunter (1964). Additionally, Leedle *et al*. (1982) suggested that osmotic shock caused by water intake and oxygen shock caused by oxygen intake with feeding should also be considered as factors that could cause a loss in viable bacteria numbers and subsequently a decrease in digestibility after feeding. Similarly, Michalowski (1977) found that a decrease in the population density of *Entodinium*- and *Diplodinium*-type ciliates occurred in Water Buffalo after feeding and attributed the decrease to dilution of the rumen contents by feed, saliva, water, and the exiting of digesta from the rumen. Therefore, it is possible that the feeding behaviour of the cows were affected by feed delivery and milking as shown by DeVries *et al*. (2003), resulting in a decrease in bacterial and ciliate numbers after feed delivery, producing the lag time in the ivSd, NDFd and Nd peaks observed. However, monitoring the feeding activity could confirm this speculation.

The effect of the interaction between the feeding treatment and the time of rumen fluid collection had no effect on the activity of lichenase, endoglucanase and protease, and had no effect on the digestibility of starch, NDF or nitrogen. For amylolytic activity, there was a significant difference in amylase activity at 23h30 between the cows fasted overnight and those fed ad lib. The lower activity observed in the fasted cows could be attributed to the absence of substrates for the amylolytic microorganisms. However, this would be expected after a few hours into the overnight fasting. Such a difference at 23h30 is therefore difficult to explain, and it is more likely related to different feeding patterns among the cows that were not monitored. For xylanase activity, the only significant difference between the cows fasted overnight and those fed *ad libitum* was observed at 11h30. This might have been caused by the different feeding behaviours displayed by the cows resulting from the treatment of the night before. Additionally, the fasting versus *ad libitum* feeding might have caused a change in the rumen-microenvironment pertaining to amylase and xylanase activity.

When fitted to a cosine function all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed, although the parameters responded differently to the feeding treatment. The effect of overnight fasting on amylase and xylanase activity produced a daily pattern with delayed acrophase and a reduced amplitude. The effect of overnight fasting on lichenase activity produced a daily pattern with a delayed acrophase and a greater amplitude. The effect of overnight fasting on endoglucanase and protease activity produced a daily pattern with an advanced acrophase and a reduced amplitude. The reason for the phase delay in endoglucanase activity observed in the *ad libitum* fed cows could be attributed to higher levels of glucose in the rumen compared to the fasted cows, as Mountfort and Asher (1985) demonstrated that glucose could play a role in the regulation of endoglucanase. Fasting the night before could have caused a change in the rumen microenvironment in relation to the *ad libitum* fed cows, producing an advanced acrophase for endoglucanase activity.

The effect of fasting on enzymatic activity varied between the substrates. Both lichenan and CMC were used to measure for endoglucanase activity. However, fasting had the opposite effect on their daily patterns. The effect of overnight feeding on ivSd and NDFd produced a daily pattern with an advanced acrophase and enlarged amplitude. The fact that the effect of fasting on Nd produced a delayed acrophase and a higher amplitude corresponds with the study by Ørskov and McDonald (1979) that showed that restricted feeding reduced the passage rate with a subsequent increase in ruminal protein degradability compared to unrestricted feeding in sheep. The reason for the variability in enzymatic activity observed for amylase, lichenase, xylanase and protease activity, and *in vitro* starch and nitrogen digestibility is not clear but might have been caused by different feeding behaviours displayed by the cows resulting from the treatment of the night before.

4.6 CONCLUSION

Patterns in activity observed for amylase, lichenase, endoglucanase and xylanase were most likely a result of feeding behaviour adapted to the time of feed delivery and milking. It was interesting to note that the protease activity was unaffected by either feeding treatment or possible feeding behaviour, although when fitted to a cosine function it did display a daily pattern that was sensitive to the availability of feed. This again confirms that different interpretations should be given to enzymatic activities and *in vitro* digestibility values. The patterns displayed by *in vitro* digestibility of starch, NDF and nitrogen, across the various fluid collection time points, were much more variable than expected, probably because various factors affect the final result, most probably related to the microenvironment within each flask, the human factor and the whole *in vitro* procedure. Such a variation would result in important consequences when computing rates of digestion, especially for starch (Hall, 2000), to be used for rationing software systems like the CNCPS (Van Amburgh *et al*., 2015). When fitted to a cosine function all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed, although the parameters responded differently to the

feeding treatment. Lactating dairy cows naturally display highest feeding activity during the day with a marked decrease in activity at night (DeVries and von Keyserlingk, 2009). However, it was apparent from the results that some feeding was continuing during the overnight period causing the differences observed in the fasted animals. Monitoring feeding behaviour influenced by fasting or *ab libitum* feeding for the overnight period could provide more insight into the patterns of enzymatic activity and *in vitro* digestibility observed.

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CHAPTER 5

GENERAL CONCLUSION

The aim of this study was to better understand the complexity of the rumen fluid in terms of enzymatic activity, to identify how methodological differences affect the enzymatic activity and in turn determine how this might affect *in vitro* digestibility measurements.

The aim of the first study was to identify an optimal method for enzymatic extraction and to explain result variability. Both blending and sonication had the ability to consistently release more amylase, lichenase, endoglucanase and xylanase enzymes. Blending is, therefore, simpler and an adequate alternative when wanting to quantify these enzymes. However, blending was not able to release more protease from the particulate matter. However, based on our results, sonication cannot be replaced by blending for the extraction of cell-associated protease enzymes. The purpose of the digestibility portion of the experiment was to define if blending was capable of improving digestibility of starch, neutral detergent fibre (NDF) and nitrogen. For the rumen *in vitro* digestibility, blending was not successful in liberating amylolytic microorganism or enzymes associated with the particulate fraction and might have negatively affected the microbial population responsible for fibre and protein digestion. Therefore, as opposed to what is sometimes suggested, blending rumen fluid before an *in vitro* digestion does not necessarily result in higher digestibility values and reduced lag (i.e. higher microbial activity) and could result in opposite results. As blending is the most common method used for extracting adhered microorganisms and enzymes in the rumen content for use in *in vitro* digestibility studies, an alternative method would need to be identified. However, sonication is not an option for extracting microorganisms and enzymes from the adhered population for *in vitro* digestibility studies, because with sonication the risk of reducing microbial activity by cell lysis exist. Therefore, according to our results, there is no procedure available at the moment that can safely

extract microorganisms and enzymes from the adherent population of rumen fluid for *in vitro* digestibility studies.

The aim of the second study was to determine if diurnal patterns in starch, NDF and protein digestibility and amylolytic, fibrolytic and proteolytic activities exist in dairy cows. By fitting the data to a cosine function, it was determined that all the parameters tested followed a daily pattern that was sensitive to the overnight availability of feed. Patterns in enzymatic activity observed for amylase, lichenase, endoglucanase and xylanase were most likely a result of feeding behaviour adapted to the time of feed delivery and milking. However, protease activity was unaffected by either feeding treatment or possible feeding behaviour. The peaks in enzymatic activity observed in the rumen fluid collection time points subsequent to feeding and milking, and the differences observed in activity between the cows fasted and cows fed *ad libitum* overnight highlighted the fact that monitoring total intake and feeding behaviour would have provided us with a better explanation of the results observed. However, due to the nature of this study, logistically investigating feeding behaviour as another parameter was not obtainable but should be considered for further studies.

The patterns displayed by *in vitro* digestibility of starch, NDF and nitrogen, across the various fluid collection time points, were much more variable than expected, probably because various factors affect the final result, most likely related not only to the dynamic complexity of the rumen but also to the microenvironment within each flask, the human factor and the whole *in vitro* procedure. Such a variation would result in important consequences when computing rates of digestion, especially for starch, to be used for rationing software systems like the CNCPS. The results obtained in this study clearly show that the substrate is not the only limiting factor during fermentation, and this is probably true for both *in vivo* and *in vitro* fermentations. For *in vitro* studies, factors such as the way we handle rumen fluid, the way we feed the animals, the time of collection of rumen fluid, the substrate we want to analyse and the diet, can affect both enzymatic activity and *in vitro* digestibility results. This is of

importance as the results obtained from *in vitro* studies are used daily to feed ruminants and to fine tune their diets.

Additionally, we speculated that analysing enzymatic activity of rumen fluid in correlation with *in vitro* digestibility studies can provide a different perspective to the changes observed in ruminal starch, NDF and protein digestibility. However, the results were not comparable. This was most likely due to the fundamental differences between the studies, as enzymatic analysis gives an instant result of what is happening in the rumen at the collection time point in terms of enzymatic activity. However, for *in vitro* assays, rumen fluid is handled for longer as it needs to be injected in all the flasks and is diluted into the medium. Results are only obtained after the incubation period; indicating the capabilities of the microorganisms sampled at the given time point to adjust to the new micro-environment and to digest a substrate. Therefore, different interpretations should be given to enzymatic activities and *in vitro* digestibility values.

For future studies, analysing for bacterial diversity using automated ribosomal intergenic spacer analysis (ARISA) should also be considered to characterise the bacterial population dynamic across a 24-hour period in relation to enzymatic activity and *in vitro* digestibility assays.